Remarks

Claims 23-25, 27, 28, 30, and 31 are pending in the subject application and currently before the Examiner. Favorable consideration of the pending claims, in view of the remarks provided herein, is respectfully requested.

As an initial matter, Applicants gratefully acknowledge the Examiner's withdrawal of the objections to the drawings and claims and certain of the rejections under 35 U.S.C. § 112, first paragraph.

The Office Action indicates that the Information Disclosure Statement (IDS) submitted 6/21/2004 is acknowledged and considered. Applicants note that the IDS was signed by the Examiner on each respective page; however, it appears that the individual references listed at pages 2-3 of the PTO/SB/08B form were not initialed as having been considered. Applicants respectfully request that the IDS be initialed and the initialed pages returned with the next Action as having been considered and made of record in the subject application.

Claims 23-25, 27, 28, 30, and 31 are rejected under 35 U.S.C. § 101 because the claimed invention is not supported by either a substantial and specific asserted utility or a well established utility. Claims 23-25, 27, 28, 30, and 31 are also rejected under 35 U.S.C. § 112, first paragraph, on the basis that one skilled in the art would not know how to use the claimed invention because it is not supported by a well-established utility or by a specific and substantial utility.

Applicants refer to M.P.E.P. §2107.01, wherein it is stated that:

> A "specific utility" is specific to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention. (...) For example, indicating that a compound may be useful in treating unspecified disorders, or that the compound has "useful biological" properties, would not be sufficient to define a specific utility for the compound. Similarly, a claim to a polynucleotide whose use is disclosed simply as a "gene probe" or "chromosome marker" would not be considered to be *specific* in the absence of a disclosure of a specific DNA target.

In the case of the currently claimed invention, the Examiner asserts that the polypeptide of SEQ ID NO: 399 has no specific and substantial utility as the specific substrate and/or the biological role is unknown. Applicants respectfully disagree with this assertion.

Applicants submit that the application provides a specific biological role and a specific substrate for the polypeptide of SEQ ID NO: 399. Applicants draw the Examiner's attention to the following paragraph 1549 of U.S. publication No. 2003/0152921:

> PAP7 [*i.e.*, the polypeptide of SEQ ID NO: 399] is known to be an important enzyme for the glycerolipid biosynthesis. In particular, PAP catalyzes the conversion of phosphatidic acid (PA) into diacylglycerol (DAG). Therefore, the biological activity of PAP7 is defined as the catalysis of said conversion reaction.

Applicants submit that the utility of a polypeptide is non-specific only if it is applicable to *any* polypeptide. In the present case, Applicants have provided both a precise biological activity and a substrate for the claimed polypeptide. This biological activity is not applicable to *any* polypeptide, although it may be common to a limited number of polypeptides displaying a PAP2 domain. Thus, the claimed polypeptide has a biologic function that is specific, namely the conversion of phosphatidic acid into diacylglycerol and the Patent Office has not produced any objective evidence that contradicts the asserted function of claimed polypeptide. Further, the product produced by PAP7, diacylglycerol, is a useful product sold by a number of commercial concerns (*e.g.*, as a research tool useful in the study of protein kinase C as indicated in the as-filed specification). Accordingly, it is respectfully submitted that the claimed polypeptide has a specific and credible utility and reconsideration and withdrawal of the rejection is respectfully requested.

Applicants also submit that no further research is needed to identify the specific substrates and diseases/disorders associated with the claimed polypeptide. Assays for determining whether a polypeptide is capable of catalyzing the conversion of phosphatidic acid into diacylglycerol are well-known in the art. Such assays for measuring phosphatidic acid phosphatase activity were available as early as 1965 (see, *e.g.*, the attached article by McCaman *et al.*, *J. Biol. Chem.*, 1965, 240:3513-3517). Applicants submit that testing the activity of the claimed polypeptide is a routine test that cannot be considered as further research.

Additionally, a number of substrates for type 2 phosphatidic acid phosphatases were known in the art at the time the subject application was filed. For example, the attached article by Kai *et al.* (*J. Biol. Chem.*, 1997, 272(39):24572-24578) teaches a number of such substrates. As indicated in

the Abstract of Kai *et al.*, PAP isozymes are known to catalyze the hydrolysis of lysophosphatidate, ceramide-1-phosphate and sphingosine-1-phosphate. Thus, many substrates for PAP isozymes were known prior to the critical date of the instantly claimed invention.

Applicants also note that the Office Action indicates that the teachings of Van de Loo *et al.*, Seffernick *et al.* and Broun *et al.* were utilized to illustrate the unpredictability of assigning function based upon structural homology and that even small structural changes can result in changes in functions for polypeptides. Applicants, again, respectfully traverse the rejection on the basis that the generalized teachings of the references are not germane to the polypeptide of SEQ. ID NO: 399 (PAP7).

Applicants respectfully submit that it was recognized in the art that PAP isozymes can have significantly divergent amino acid sequences. For example, the two PAP isozymes identified by Kai *et al.* share only 47% identity, yet each of the polypeptides are classified in the PAP family (see paragraph bridging pages 24574-24575). The attached article by Roberts *et al.* (*J. Biol. Chem.*, 1998, 273(34)22059-22067) teaches a third member of the PAP family (identified as PAP-2c) that has 54% and 47% sequence homology to the PAP isozymes of Kai *et al.* (see Abstract and pages 22061-22062, Results, *Sequence Comparison of PAP2a, -2b, and* -2c). As is apparent from the teachings of these references, PAP isozymes can have significantly divergent amino acid sequences while retaining the catalytic activity characteristic of PAP isozymes. Thus, it is respectfully submitted that members of the same family of proteins, in this case PAP isozymes, can exhibit significant differences in amino acid sequence and still be members of the same enzymatic family.

Applicants, again, respectfully submit that the identification of the claimed polypeptide as a member of the PAP2 family is not based on sequence alignment alone. Rather, Applicants have identified a Pfam domain using Hidden Markov Modeling (HMM) analysis, not sequence alignment (the results of which are attached as Appendix 1 of the response filed 22 December 2003). As indicated therein, the e-value obtained for the PAP2 Pfam domain present in SEQ. ID NO: 399 is $8.6e^{-14}$, which is a highly significant score. Accordingly, one of skill in the art would reasonably believe that SEQ. ID NO: 399 belongs to the PAP2 superfamily as was asserted in the originally filed application.

In addition Applicants submit that SEQ. ID NO: 399 not only displays a PAP2 Pfam domain, but also displays the following conserved phosphatase signature motif: T-X_6-RP-X_{34}-PSGH-X_{48}-SR-X_5-H-X_3-D. As taught by Stukey *et al.*, this conserved motif can serve as a predictor of phosphate enzyme function (see page 471, column 1, line 42-43 of Stukey *et al.*).

In Stukey *et al.* it is stated that:

> The following existing experimental evidence supports the identification of this phosphatase signature motif:
>
> a) These conserved sequences are conceptually present in 13 known phosphatases; (...)
>
> b) Structure function analysis of the human glucose-6-phosphatase indicates two of the absolutely conserved residues *[the arginine and the histidine highlighted by a box in the above consensus sequence]* are essential for enzyme function; and
>
> c) A current database "hypothetical" gene product (Accession No. U51031) having this phosphate signature motif has been identified as a diacylglycerol pyrophosphate phosphatase. (see page 471, column 1, lines 47-60 of Stukey *et al.*)

Accordingly, in view of the presence of (1) a highly significant PAP2 Pfam domain; and (2) a phosphatase signature motif displaying the two absolutely conserved residues essential for enzyme function, one of skill in the art would reasonably conclude that SEQ. ID NO: 399 is a phosphatidic acid phosphatase (PAP).

In addition, Applicants submit that numerous methods of using phosphatidic acid phosphatases are known and well-established in the art. For example, U.S. Patent No. 6,242,179 (submitted in the Information Disclosure Statement dated December 22, 2003) describes the treatment of immune disorders and diseases associated with cancer using the HPA-1 phosphatidic acid phosphatase; U.S. Patent No. 5,100,787 describes a method for preparing highly purified phosphatidylinositol using phosphatidic acid phosphatases; and U.S. Patent No. 6,476,294 describes a method of modifying the lipid composition in a plant cell using phosphatidic acid phosphatases.

Applicants also respectfully submit that the claimed polypeptide also has a specific, credible and/or well established utility in view of the teachings of the specification at paragraph 1550 and 1555 of United States Patent Application Publication No. 2003/0152921 where it is indicated that PAP7 expression is suppressed in tumor tissues and that PAP7 polypeptides, or fragments thereof, can be used to generate antibodies using any technique known to those skilled in the art and used to identify tissues and diagnose PAP7-related disorders, including tumors. Paragraph 1555 further indicates that antibody mediated detection of PAP7 polypeptides can accomplished by well-known methods, such as immunohistochemistry or ELISA. Thus, it is respectfully submitted that one skilled in the relevant art would have recognized that one could use PAP7 to identify tumor tissue on the basis of suppressed expression of PAP7 as compared to normal tissue. In view of such a teaching, it is respectfully submitted that one skilled in the relevant art would have recognized that PAP7 (SEQ. ID NO: 399) has a specific, credible, and well-established utility as a tumor marker (*e.g.*, tumor cells contain reduced amounts of PAP7 as compared to normal tissues) and that one skilled in the art would have known how to use PAP7 for the evaluation of tissue and tumor samples. Accordingly, reconsideration and withdrawal of the rejections set forth under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph is respectfully requested.

It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position. Applicants expressly reserve the right to pursue the invention(s) disclosed in the subject application, including any subject matter canceled or not pursued during prosecution of the subject application, in a related application.

In view of the foregoing remarks, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

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FCE/sl
Attachments: Copy of McCaman *et al.* (1965) reference
Copy of Kai *et al.* (1997) reference
Copy of Roberts *et al.* (1998) reference

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Intermediary Metabolism of Phospholipids in Brain Tissue

II. PHOSPHATIDIC ACID PHOSPHATASE*

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Phosphatidic acid phosphatase, first studied in particulate fractions of liver (1), has also been found in brain tissue (1–3). The key role of this enzyme in the biosynthesis of glycerophosphatides was suggested by the observation that D-1,2-diglycerides (the products of the enzyme reaction) serve as precursors for the biosynthesis of phosphatidylcholine and phosphatidylethanolamine (4). Since previous studies[1] from this laboratory have shown that these phospholipids and certain enzymes associated with their metabolism show marked changes associated with alterations in myelin (5–8), a study of phosphatidic acid phosphatase in normal and demyelinating nervous tissue was undertaken.

The present report describes a micromethod for the determination of phosphatidic acid phosphatase activity applicable to microgram and submicrogram samples of nervous tissue. The distribution of phosphatidic acid phosphatase activity in (*a*) brain samples during early postnatal development, (*b*) peripheral nerve and central white tract undergoing demyelination, and (*c*) discrete morphological subdivisions of adult rabbit brain is reported.

EXPERIMENTAL PROCEDURE

Materials—The phosphatidic acids used in this study were prepared from a variety of lecithins. A lecithin fraction was obtained from bakers' yeast[2] according to the procedure of Hanahan and Jayko (9). The yeast lecithin and a commercial preparation of soy lecithin were subjected to repeated precipitations from ether with acetone (8 volumes excess) and then chromatographed on alumina (10). In addition, a "chromatographically pure" commercial egg lecithin preparation (General Biochemicals) was used directly as indicated below.

Chromatography on silicic acid paper (11) indicated that these compounds were homogeneous, and quantitative analysis of choline (12), ester (13), and phosphate (14) gave results within 10% of the expected ratios of 1:2:1. The purified lecithins were then converted to the corresponding phosphatidic acids with either carrot chromoplasts (15) or a commercial phospholipase C preparation from cabbage (General Biochemicals). The use of the commercial enzyme afforded greater yields and greatly reduced the contamination by carotenoids and phospholipids present in the chromoplast preparations, thus facilitating the purification of the phosphatidic acids. The phosphatidic acids, purified and converted to the sodium salts according to the procedure of Kates (15), were free of inorganic phosphorus and choline and had an acyl ester to phosphate ratio of between 1.92 and 2.05. A sample of DL-phosphatidic acid (dioleyl) was generously supplied by Dr. R. W. Albers. The esterified fatty acids in the various preparations were converted to the methyl esters with the use of methanol-sulfuric acid (16). The fatty acid composition of the final preparations is listed in Table I.

Methods—Buffer-substrate, 10 μl consisting of 4 mM phosphatidic acid in 0.1 M Tris buffer, pH 7.4, was added to an ice-cold microtube containing 1 μl of homogenate or the lyophilized tissue sample. After incubation at 38° (usually for 30 min), the reaction was stopped with the addition of 10 μl of 0.6 N trichloroacetic acid. After centrifugation, a 15-μl aliquot was removed and placed in a tube (10 × 7.5 mm) containing 1 ml of color reagent for inorganic phosphate. After incubation of the samples for 20 min at 38°, the optical density was determined at 820 mμ. The color reagent is essentially that described by Buell *et al.* (17) and is prepared just before use by dissolving 300 mg of ascorbic acid in 12 ml of water and adding 0.3 ml each of 10 N H_2SO_4 and 5% ammonium molybdate in water. The molar extinction coefficient for inorganic phosphate is 25,500. Standards (1 μl of 5 mM KH_2PO_4) instead of tissue were included in every determination. In addition tissue blanks (tubes containing substrate in buffer carried through the incubation and acidified before the addition of tissue) were included as a control for inorganic phosphate present in the homogenates and sections. The sensitivity of the method may be enhanced 10- to 20-fold by reduction in the volume of the color reagent and a corresponding increase in the concentration of the reactants so that their final concentrations are comparable to those described above.

The techniques for the preparation and handling of tissues have been previously described (6). Protein was determined by the method of Lowry *et al.* (18). The statistical treatment of the data for the developing brain studies was performed in accord with published procedures (19).

RESULTS

The effect of varying the pH from 4.6 to 9.0 was studied for each of the phosphatidic acid preparations with the use of rabbit and rat brain homogenates. In all cases there was an apparent optimum in the region of pH 7.0 to 7.4 (Tris buffer), but even

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[1] The preceding paper of this series (5) describes the distribution of choline phosphokinase within various areas of the nervous system and the changes in the activity of this enzyme during demyelination.

[2] Generously supplied by Standard Brands, Inc.

TABLE I

*Fatty acid composition of phosphatidic acids**

The gas-liquid chromatography was performed in a dual column instrument (Packard) at 195° with diethylene glycol succinate (15% on Chromosorb W) and Apiezon L (10% on Chromosorb W). The values indicate the relative weight of each constituent.

Fatty acid†	Phosphatidic acid			Synthetic (dioleyl)
	Soybean	Yeast	Egg	
	%	%	%	%
14:0				5
16:0	16	10	36	6
16:1		70		11
18:0	4		14	
18:1	10	20	36	78
18:2	70		14	

* The preparation of these materials from lecithins isolated from the indicated sources (soybean, yeast, egg) is described in text.

† Although traces of methyl esters of other fatty acids were detected, the above accounted for 90 to 98% of the total observed.

at pH 6.0 (dimethylglutarate) and 8.5 (Tris buffer) the activity was still 90% of the maximum observed. At pH 5.1 (acetate) and 9.0 (2-amino-2-methyl-1,3-propanediol) the enzyme activity was reduced 65% and 25%, respectively.

The results obtained when the substrate concentration of each of the phosphatidic acid preparations was varied over a wide range were also similar, so that only the results obtained with the yeast preparation are given (Table II). The enzyme appeared to be saturated at phosphatidic acid concentrations of 5.0 to 6.0 $\times 10^{-3}$ M, depending on the particular preparation. The apparent K_m values ranged from 2.2 $\times 10^{-4}$ M to 5.2 $\times 10^{-4}$ M. The above statements with respect to enzyme saturation or apparent K_m can, at best, be only approximate, since it is not known whether the phosphatidic acids, even as the sodium salt, form a true solution. The enzyme activities of rat brain homogenates with yeast, soy, and egg phosphatidic acids were roughly equivalent (77 to 87 μmoles per g per hour) while the activity with the phosphatidic acid containing two oleyl groups was approximately 50% lower (40 μmoles per g per hour).

The reaction rates at various temperatures relative to 38° were 5, 45, 160, and 159% at 0, 23, 45, and 55°, respectively. The liberation of inorganic phosphorus was proportional to the amount of tissue over a wide range (10 to 150 μg) and to time (up to 60 min).

As reported by Smith, Weiss, and Kennedy (1), we found magnesium and calcium ions in excess of 1 $\times 10^{-3}$ M to be extremely inhibitory. On the other hand, KCl and NaCl had no effect up to 0.2 M. Versene and KF up to 1 $\times 10^{-3}$ M were without effect, but a 15 and 22% inhibition was observed when their concentration was increased to 1 $\times 10^{-2}$ M. Ouabain (5 $\times 10^{-4}$ M) and mercaptoethanol (up to 2.5 $\times 10^{-2}$ M) were without effect. Cutscum (Fisher Scientific Company), Tween 20, and Triton X-100 at concentrations above 0.1% all showed marked inhibition.

A study of the enzyme activity in early postnatal rabbit brain samples is given in Table III. It was of some interest not only to study the variation as a function of age, but also (with a given animal) to compare the activities of two areas having different rates of development. Since the cortex and medulla-pons have been previously shown to have widely separated rates of biochemical maturity (20), these areas were selected for this study.

The activity of the cortex samples reached a maximum at 20 days of age (90% higher than the 4-day sample) and then decreased with increasing age. The trend over days is highly significant ($p < 0.005$) for the 4- to 20-day increase, as is the subsequent decrease ($p < 0.01$) with maturity. The activity of the medulla-pons is initially higher than the cortical samples and shows no significant trend with age ($p < 0.5$) up to 32 days of age. The activity in the adult medulla is significantly lower ($p < 0.01$) than that at 32 days. Except for the decreased activity in the adult, the changes in the above activities of both areas show a fair correlation with the degree of myelination observed histochemically (21) and with the deposition of phospholipids (20) at the various ages. It is interesting to note that the net increases in the content of phospholipids for the period

TABLE II

Effect of substrate concentration on brain phosphatidic acid phosphatase

Yeast phosphatidic acid at the indicated concentrations in 0.1 M Tris buffer (pH 7.4) was used in both experiments. The indicated activities were obtained with rat brain homogenates.

Substrate concentration	Activity
mM	*μmoles/g/hr*
Experiment 1	
2.0	84.5
4.1	83.9
8.2	86.3
12.3	69.0
Experiment 2	
0.045	16.5
0.091	21.0
0.27	57.6
0.44	71.5
0.64	74.5
0.95	76.4
2.3	83.0
5.1	87.0

TABLE III

Phosphatidic acid phosphatase activity of rabbit brain during early postnatal development

These activities were obtained with yeast phosphatidic acid (3.5 mM) in 0.1 M Tris, pH 7.4. Each value represents the mean of triplicate determinations on homogenates from two separate animals at each age.

Age	Activity	
	Cortex	Medulla-pons
days	*μmoles/g/hr*	
4	25.0	60.5
8	30.0	55.2
12	33.0	59.6
16	36.2	55.8
20	47.5	53.6
32	41.8	53.6
Adult	35.4	30.0

from 3 to 32 days of age are 8 and 32 μmoles per g, wet weight, for the cortex and medulla, respectively (20). With these values, the rates of synthesis for the 29-day period would be approximately 0.01 and 0.05 μmole per g per hour, respectively. If one assumes that phosphatidic acid phosphatase is an obligatory enzyme in the synthesis of the bulk of these phospholipids, then the minimal activity *in vitro* of this period (Table III) is at least 1000 times greater than the above rates.

A further association between changes in phosphatidic acid phosphatase activity and alterations in the degree of myelination was obtained by studying tissues in which demyelination is a significant phenomenon (Table IV). After surgical section, the phosphatidic acid phosphatase activity in the distal degenerating portion of a peripheral nerve (tibial) and a central white tract (optic) is elevated. The activities of the degenerating tibial nerve are markedly higher than the control at all times with a maximum at 14 days (350% of that of the control). The increase in activity of the degenerating optic nerve is not apparent until 45 days and is less marked (147% of that of the control) than that of the tibial. The maximum enzyme activities of the two nerves occur at a time which coincides with the time of active demyelination as judged by morphological and chemical criteria (8). Further confirmation of an elevated enzyme activity in demyelinating nervous tissues was obtained in a preliminary study of the effects of x-irradiation on rat nervous tissue metabolism. Six to seven months after x-irradiation (3500 rads of 250-Kv x-ray), plaques of demyelination are observed in the ventral and lateral columns (white matter) of a high percentage of animals (22). Histological examination of the 7-month irradiated spinal cord sample used in this study[3] showed major areas of advanced demyelination specifically in the ventral and lateral columns. These two areas also have an elevated enzyme activity (approximately 200% of that of the control) in contrast to the dorsal column, which shows no lesion and no change in enzyme activity. A slight increase was also observed in the activity of the irradiated grey matter (136% of that of the control).

A quantitative histochemical study of various areas of the nervous system (Table V) indicates that the layers rich in grey matter have higher activities than the subjacent white matter. The grey matter of the cortex, cerebellum, and spinal cord is approximately twice as active as the corresponding white matter. Similarly, the trigeminal nerve ganglion cells are more active than the peripheral or central portion of the nerve fibers. The high enzyme activity of the area postrema is of particular interest in that this structure is generally devoid of the neuronal soma that characterize the other structures of high activity. Since a high turnover of phosphatidic acid has been shown to be associated with secretory activity in a variety of cells (23), the high enzyme activity of this area is compatible with the suggestion that it may have a neurosecretory function (24).

Preliminary studies of the distribution of phosphatidic acid phosphatase activity in fractions isolated by differential centrifugation techniques using a 10% homogenate of rat brain in 0.32 M sucrose, gave the following results. (*a*) Approximately 96% of the total activity is particulate (pellet, 100,000 × *g* for 30 min); (*b*) more than 95% of the particulate activity was found in the "mitochondrial" (12,000 × *g* for 20 min) and "microsomal" (100,000 × *g* for 30 min) fractions; and (*c*) the activity in the mitochondria fraction was only a little less than that of the microsomal fraction (37% and 48%, respectively, of the activity in the original homogenate), but the specific activity of the latter was twice that of the mitochondrial fraction. While these studies confirm previous observations on the particulate nature of this phosphatase in brain (3), no conclusive information is currently available concerning the localization of this enzyme in neural tissue in well defined subcellular fractions. More elaborate studies are currently in progress concerning the subcellular localization of phosphatidic acid phosphatase in neural tissue.

[3] Unpublished results. We are indebted to Dr. A. Carsten, Brookhaven National Laboratories, and to Dr. W. Zeman, Department of Pathology, Indiana University School of Medicine, for making this material available to us.

TABLE IV

*Phosphatidic acid phosphatase activity in Wallerian degeneration**

These results were obtained on lyophilized samples of rabbit nervous tissue with the use of the dioleyl phosphatidic acid as the substrate. Other experimental detail is given in the text. Each value represents the mean of three to four determinations on material from one animal at each time period.

Days of degeneration	Activity	
	Tibial nerve	Optic nerve
	μmoles/g (dry wt)/hr	
0	52	141
14	180	145
45	142	207
100	167	163

* This material was obtained as described previously (4).

TABLE V

Phosphatidic acid phosphatase in rabbit brain sections

Synthetic phosphatidic acid (dioleyl) was used as the substrate. Each value represents the mean of three to seven determinations on tissue from one animal.

Tissue	Activity
	μmoles/g (dry wt)/hr inorganic phosphate
Cerebellum	
Molecular	211 ± 15
Granular	210 ± 14
White	83 ± 4
Parahippocampal cortex	
Molecular	253 ± 23
Neuronal	176 ± 4
White	117 ± 9
Caudate	139 ± 9
Vagus nerve	142 ± 15
Dorsal root ganglion	425 ± 36
Area postrema	498 ± 90
Spinal cord	
Grey	246 ± 72
White	95 ± 30
Trigeminal nerve	
Central	89 ± 10
Peripheral	106 ± 15
Ganglion	269 ± 23

DISCUSSION

Our results would indicate that enzymatically prepared phosphatidic acids from a variety of natural sources serve as active substrates for phosphatidic acid phosphatase in rat and rabbit nervous tissues. Although there was considerable variation in the fatty acid composition of these preparations, no specific relationship was observed between the enzyme activity and the fatty acid composition. The synthetically prepared DL-phosphatidic acid containing 2 oleic acid residues was the least active. Other workers have also found that synthetically prepared phosphatidic acids incubated with brain homogenates were hydrolyzed to a lesser extent than the phosphatidic acids prepared from natural sources (2, 3, 25).

The elevated activity of phosphatidic acid phosphatase in nervous tissue undergoing demyelination is consistent with an increased incorporation of ^{32}P- and ^{14}C-labeled choline and cytidine intermediates into the glycerophosphatides of degenerating peripheral nerve (26–28). The increasing enzyme activity in the cortex up to 20 days after birth (Table III) is complementary to studies showing an increase in the content of glycerophosphatides (29) and in the labeling of glycerophosphatides from various ^{14}C- and ^{32}P-labeled intermediates at various stages of development of the brain (30). Although it is tempting to suggest that such increases in enzymatic activity in demyelinating tissues and during development may be directly related to increased synthesis of glycerophosphatides, there are a number of discordant aspects of such a relationship. For example, although the enzyme activity in demyelinating tissue is increased, the concentration of glycerophosphatides of these tissues is markedly reduced (6). In addition, the decrease in enzyme activity, after 30 days of age, in the two areas of brain studied, does not parallel the further increase in the content of glycerophosphatides in these areas during the same period (20). And finally, the activity of the grey matter of nervous tissue is generally higher than that of the lipid-rich white matter (Table V). However, if one assumes that the enzymes responsible for biosynthesis of glycerophosphatides are under the control of a "feedback" mechanism which regulates their synthesis, then the disparity of the above observations may be resolved. Thus, the accumulation of an appreciable concentration of glycerophosphatides in conjunction with myelination in the developing brain (particularly in the white matter) might then bring about the observed reduction of enzyme activity. Similarly, the increased enzyme activity of demyelinating nervous tissue and in grey matter may be explained on the basis of reduced content of glycerophosphatides in these tissues. A mechanism similar to the above has been previously suggested for the regulation of cholesterol biosynthesis in nervous tissue (31).

Studies of Wilgram and Kennedy (32) suggest that phosphatidic acid phosphatase activity in liver may be associated with a subcellular fraction having sedimentation characteristics similar to those of lysosomes. If the brain enzyme were also lysosomal, another interpretation of the present data would be possible on the basis of information currently available concerning the distribution of lysosomes in neural tissues and their alterations in pathological tissues. Koenig (33) has reported that lysosomes are more plentiful in grey than in white matter of the brain (*cf.* distribution of enzyme, Table V) and that there is an increase in number and size of lysosomes in reactive neuroglia and lipid phagocytes (*cf.* increase of enzyme activity, Table IV). It is thus possible that the distribution of phosphatidic acid phosphatase in adult nervous tissue corresponds to the distribution of specific cytoplasmic organelles. Further, the fluctuations of the activity of this enzyme observed in the demyelinating tissues may be a function of changes in the number or character of such organelles.

SUMMARY

A procedure has been described for a rapid and sensitive assay of phosphatidic acid phosphatase activity in neural tissue. It was observed that phosphatidic acids biosynthetically prepared (with phospholipase C) from lecithins isolated from various natural sources (yeast, egg, and soy bean) serve as active substrates for the brain enzyme.

In early postnatal samples of cortex of rabbit brain, there is a progressive increase in enzyme activity from 4 to 20 days of age. The activities of samples from the medulla-pons from the same animals exhibit a slight reduction during this period. In samples from both areas of the adult brain the enzyme activity is significantly lower than that in the corresponding postnatal samples.

The enzyme activities of nervous tissues undergoing active demyelination were 2- to 3-fold higher than the levels in comparable normal tissues. A study of the distribution of enzyme activity in discrete morphological subdivisions of the adult rabbit brain indicates that the neuron-rich grey structures are 2- to 4-fold more active than the adjacent white matter. Consideration has been given to the possibility that the observed distribution and fluctuations of the activity of phosphatidic acid phosphatase in neural tissue may be related to (*a*) the regulation of the enzyme activity by a "feedback" mechanism responsive to the glycerophosphatide content of the tissues involved, or (*b*) the association of the enzyme activity with a specific cytoplasmic organelle which undergoes changes corresponding to those observed in the present study.

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Cloning and Characterization of Two Human Isozymes of Mg^{2+}-independent Phosphatidic Acid Phosphatase*

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We obtained two human cDNA clones encoding phosphatidic acid phosphatase (PAP) isozymes named PAP-2a (M_r = 32,158) and -2b (M_r = 35, 119), both of which contained six putative transmembrane domains. Both enzymes were glycosylated and cleaved by *N*-glycanase and endo-β-galactosidase, thus suggesting their post-Golgi localization. PAP-2a and -2b shared 47% identical sequence and were judged to be the human counterparts of the previously sequenced mouse 35-kDa PAP(83% identity) and rat Dri42 protein (94% identity), respectively. Furthermore, the sequences of both PAPs were 34–39% identical to that of *Drosophila* Wunen protein. In view of the functions ascribed to Wunen and Dri42 in germ cell migration and epithelial differentiation, respectively, these findings unexpectedly suggest critical roles of PAP isoforms in cell growth and differentiation. Although the two PAPs hydrolyzed lysophosphatidate and ceramide-1-phosphate in addition to phosphatidate, the hydrolysis of sphingosine-1-phosphate was detected only for PAP-2b. PAP-2b was expressed almost ubiquitously in all human tissues examined, whereas the expression of PAP-2a was relatively variable, being extremely low in the placenta and thymus. In HeLa cells, the transcription of PAP-2a was not affected by different stimuli, whereas PAP-2b was induced (up to 3-fold) by epidermal growth factor. These findings indicate that despite structural similarities, the two PAP isozymes may play distinct functions through their different patterns of substrate utilization and transcriptional regulation.

Phosphatidic acid phosphatase (PAP)[1] (EC 3.1.3.4) supplies diacylglycerol (DG) in glycerolipid biosynthesis by dephosphorylating phosphatidic acid (PA) (1). Since both DG (2) and PA (3) are potent signaling molecules, PAP plays an important role in cellular signal transduction in addition to lipid biosynthesis (4). In signaling systems mediated by phospholipase D (5), liberated PA is generally converted, albeit to different extents, to DG by the action of PAP (6–8), thus suggesting that PAP contributes to the control of the relative balance of the two lipid second messengers.

There exist two forms of mammalian PAP that can be distinguished with respect to subcellular localization and enzymologic properties (9). So far, distinct functions have been ascribed to the two forms of PAP. The type 1 PAP, a Mg^{2+}-dependent and *N*-ethylmaleimide-sensitive enzyme, is considered to be primarily involved in lipid synthesis based on its translocation from the cytosol to microsomes upon stimulation of cellular triacylglycerol synthesis (10–12). In this context, a recent work showed using a novel enzyme inhibitor that the type 1 PAP indeed participated in triacylglycerol synthesis in mouse macrophages (13). On the other hand, the type 2 PAP, a Mg^{2+}-independent and *N*-ethylmaleimide-insensitive enzyme, has been postulated to participate in cellular signal transduction mediated by phospholipase D. The finding that the activities of the type 2 PAP and phospholipase D were decreased in a coordinate manner in transformed fibroblasts (14) is consistent with the role of this isoform in signal transduction. However, such distinct functions ascribed to the PAP isoforms need to be further studied, since there are several reports describing the participation of the type 1 rather than the type 2 PAP in signal transduction occurring in, for example, polymorphonuclear leukocytes stimulated with inflammatory substances (15) and EGF-stimulated A431 cells (16).

A novel aspect of the function of the type 2 PAP has recently been disclosed by its broad substrate specificity. The enzyme purified from rat liver could hydrolyze ceramide-1-phosphate and sphingosine-1-phosphate in addition to PA and lyso-PA (17). Since ceramide, sphingosine, and their phosphorylated derivatives are known to serve as signaling molecules (18–20), the type 2 PAP can be involved in the metabolic processing of lipid mediators derived from both glycerolipids and sphingolipids. Furthermore, the type 2 PAP from rat liver has been described to be capable of hydrolyzing DG pyrophosphate (21), a novel phospholipid with a potential signaling function at least in plants (22).

Despite the apparent importance of PAP as noted above, relatively little has been known for the molecular structure of this enzyme. After several attempts of enzyme purification of the type 2 PAP (23–26), we have finally succeeded in the cDNA cloning of a mouse 35-kDa enzyme, which was confirmed to be the type 2 plasma membrane-bound PAP (27). The cloned enzyme is a novel glycoprotein exhibiting a channel-like structure with six putative transmembrane domains. We soon noticed (28) that the amino acid sequence of the mouse PAP is 34 and 48% identical to those of *Drosophila* Wunen protein (29) and rat Dri42 protein (30), respectively. The Wunen and Dri42 have been cloned as gene products involved in the regulation of germ cell migration (29) and in epithelial differentiation (30), respectively. However, it is not determined whether the Wunen and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB000888 and AB000889.

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[1] The abbreviations used are: PAP, phosphatidic acid phosphatase; DG, diacylglycerol; PCR, polymerase chain reaction; EGF, epidermal growth factor; RACE, rapid amplification of cDNA ends; HA, influenza virus hemagglutinin; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).

Dri42 possess the PAP catalytic activities. The rat Dri42 was described to reside in the endoplasmic reticulum (30), whereas the mouse 35-kDa PAP (with a closely related structure) was confirmed to localize in the plasma membranes (27). Furthermore, the purification of the type 2 PAP yielded enzyme proteins with variable molecular sizes (24–26), thus suggesting the presence of multiple forms of the type 2 PAP. The systematic characterization of the type 2 PAP isozymes should help to understand the biological implications of this novel type of phosphatase. We thus attempted to clone the type 2 PAP isozymes. We have cloned two human PAP-2 isozymes designated 2a and 2b, which happened to be the human counterparts of the mouse 35-kDa PAP and rat Dri42, respectively. The two PAP isozymes showed distinct properties despite their structural similarities, suggesting their different functions.

EXPERIMENTAL PROCEDURES

Materials—The sources of most of the materials have been described previously (23, 27, 31). [γ-^{32}P]ATP and [α-^{32}P]dCTP were obtained from Amersham Corp., and [^{35}S]methionine was from NEN Life Science Products. Sphingosine, long chain ceramide, sphingomyelin, dioleoylglycerol, monooleoylglycerol, and phospholipase D (type VI, from *Streptomyces chromofuscus*) were obtained from Sigma. EGF was obtained from Collaborative Research. *Escherichia coli* DG kinase was obtained from Calbiochem, and the protein assay kit was from Pierce. *N*-Glycanase was obtained from New England Biolabs, and sialidase and endo-β-galactosidase were the products of Seikagaku (Tokyo, Japan). The rabbit anti-calnexin antibody has been described (32). The rhodamine-conjugated anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories Inc., and anti-HA epitope tag antibody, 12CA5, was obtained from Boehringer Mannheim. Several cell lines, 293, HeLa, and HepG2, were cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) as described previously (27, 31).

Preparation of Lipid Substrates—Radiolabeled PA, lyso-PA, and ceramide-1-phosphate were prepared by incubating dioleoylglycerol, monoolein, and ceramide, respectively, with [γ-^{32}P]ATP and *E. coli* DG kinase (33) as described previously (23, 27). [^{32}P]Sphingosine-1-phosphate was prepared by acid hydrolysis of labeled ceramide-1-phosphate as described by Gomez-Munoz *et al.*(34). The labeled lipids were purified by thin layer chromatography and quantitated by phosphate analysis and radioactivity determination. Nonlabeled ceramide-1-phosphate was prepared by phospholipase D treatment of sphingomyelin as described by Imamura and Horiuti (35).

Lipid Phosphate Phosphatase Assay—The specific radioactivities of the labeled substrates were 5,000–20,000 cpm/nmol, and the substrates were added as Triton X-100 mixed micelles. The phosphatase activities hydrolyzing different substrates were measured under the same conditions as described previously (23, 27). The reaction mixture (50 μl) contained 50 mM Tris-HCl (pH 7.5), 0.2 mM labeled lipids, 3.2 mM Triton X-100, 1 mM EDTA, bovine serum albumin (1 mg/ml), and enzyme. Except for incubation with sphingosine-1-phosphate, the reaction was continued for 5 min at 37 °C, and the liberation of water-soluble $^{32}P_i$ was determined by liquid scintillation counting as described before (23, 27). The incubation with sphingosine-1-phosphate was continued for 20 min, and the reaction was stopped with 10 μl of concentrated formic acid as described by Waggoner *et al.*(17). In this case, the released $^{32}P_i$ was separated by thin layer chromatography (17). All reaction rates were proportional to the amounts of enzyme protein and incubation time and were usually done in triplicates, the results of which differed <5%.

PCR Amplification of Human cDNAs Encoding PAP Isozymes—We initially searched GenBank™ data base for the human cDNA sequences encoding proteins homologous to the mouse PAP (27). We found two representative cDNA fragments (accession numbers H68363 and N68923) encoding the partial C-terminal amino acid sequences, which were distinct from each other but similar to the corresponding regions of the mouse PAP. We presumed that these cDNAs represented the clones encoding PAP isozymes and tentatively designated the predicted proteins as PAP-2a and -2b. To obtain the cDNAs containing the complete coding regions for the putative PAP isozymes, we made use of the RACE-PCR method using Marathon cDNA amplification kit from CLONTECH. We first synthesized oligoprimers HPa0 and HPb0 based on the sequences unique to the PAP-2a (accession number H68363) and PAP-2b (accession number N68923) cDNAs, respectively. Using these primers, the first-strand cDNAs were synthesized from 2 μg of total RNA prepared from HepG2 cells as described previously (31). The first-strand cDNAs thus synthesized were subjected to the first round of RACE-PCR using other nested primers, each specific for PAP-2a and -2b (HPa1 and HPb1, respectively) following the manufacturer's protocol. The amplified fragments (922 bp for PAP-2a and 820 bp for PAP-2b) were gel-purified, subcloned into pBluescript IISK+ (Stratagene), and sequenced using ABI PRISM system (Perkin-Elmer). The next and final round of RACE-PCR amplification of the two cDNAs was then performed using specific primers (HPa2 and HPb2) synthesized on the basis of the determined nucleotide sequences. The RACE-PCR amplification described resulted in the sequencing of 937 and 1024 bp of cDNAs containing the complete coding regions for PAP-2a and PAP-2b, respectively. In the final experiments, we synthesized the sense-directed primers, HPa3 and HPb3, based on the 5′-most sequences of the cDNAs sequenced for PAP-2a and 2b as described above. The full-length cDNAs encoding PAP-2a and PAP-2b were then obtained by reverse transcriptase-PCR from 2 μg of total HepG2 RNA using Superscript preamplification system (Life Technologies, Inc.). In this case amplification was done as described previously (31), using the primer sets of HPa1-HPa3 and HPb1-HPb3 for PAP-2a and PAP-2b, respectively. The resultant PCR products were subcloned into pBluescript II SK at the *Eco*RV site, and the sequences of the two cDNAs were confirmed by sequencing five independent clones for each. DNA sequences were analyzed and aligned as described previously (27, 36). The primers used in these experiments are summarized as follows (5′ → 3′). The corresponding nucleotide numbers in the respective cDNA clones are shown in the parentheses. For amplification of PAP-2a cDNA: HPa0, AAGGCTTGTACACCAGGAAGAAAG (for synthesizing the first-strand cDNA); HPa1, AACAGGCCAGCTTCAGGTGGGCAC (937–914); HPa2, GTAAGGGTACTTGATGGACTCATC (194–171); and HPa3, ACCGCAGCTCAGTCCATCGCCCTTG (1–25). In the case of sequencing PAP-2b: HPb0, AGTCGGGCAAAAGTTTTTCCCTAC (for synthesizing the first-strand cDNA); HPb1, TACTGTCTGATGAGATTGGAGAGC (1024–1001); HPb2, TCACCAGTTTTCAGTGGGTACTTG (248–225); and HPb3, AGCGCCATGCAAAACTACAAGTAC (1–24).

Construction of Expression Plasmids—The PAP cDNAs were subcloned into pREP9 (Invitrogen) at the *Hin*dIII-*Bam*HI site as described previously (27). To express PAP isozymes as GFP-fusion proteins, two primers for each cDNA were synthesized for PCR amplification. In the case of PAP-2a, the sense primer, HPa-N, 5′-GGCAGCCCGGGCTAGCACCATGTTCGACAAGAC-3′ (corresponding to nucleotides 29–61) was designed to generate a *Nhe*I site (underlined). The antisense primer, HPa-C, 5′-GCACCCTGCTGCGTCGACAGGCTGGTGATTG-3′ (nucleotides 917- 887) was designed to introduce a *Sal*I site and also to destroy the termination codon of PAP-2a (underlined). PCR amplification was performed as described previously (31) using the two primers and the PAP-2a cDNA in pBluescript as a template. The amplified fragment was digested with *Nhe*I and *Sal*I and subcloned into the plasmid pEGFP-3N (CLONTECH) at the corresponding site. A similar strategy was adopted for the construction of the expression plasmid of PAP-2b-GFP fusion protein. In this case, the PCR mixture contained the sense primer, HPb-N (5′-CGATAAGCTTGCTAGCGCCATGCAAAAC-3′, corresponding to nucleotides 1–15 in the PAP-2b cDNA, restriction site and mutated nucleotides underlined), the antisense primer, HPb-C (5′-AGGAGGTGGGTGCTCGAGACATCATGTTGTG-3′, nucleotides 958–928), and the PAP-2b clone in pBluescript. The amplified fragment was digested with *Nhe*I and *Xho*I and subcloned into the corresponding site of the plasmid pEGFP-3N.

The plasmids for expression of the two PAPs each with an HA epitope tag (YPYDVPDYA) at the C terminus (PAP-HA) were prepared from the pEGFP-3N expression vectors constructed as described above. For this purpose, we prepared an adaptor coding for a HA tag as follows. The two oligonucleotides, 5′-TCGAGTATCCATATGATGTTCCAGATTATGCTCTCGACGCGCGGGCC-3′ and 5′-CGCGCGTCGAGAGCATAATCTGGAACATCATATGGATAC-3′ at 50 μM each were dissolved in the annealing buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 100 mM NaCl. The mixture was heated at 90 °C for 2 min and cooled slowly to 50 °C at the rate of 1 °C/min. The annealed adaptor was inserted into the PAP-2a/pEGFP-3N clone at the *Sal*I-*Apa*I site. The vector expressed PAP-2a with the C-terminal HA tag with 14 additional vector-derived residues. The expression vector for PAP-2b with a HA tag was constructed by a similar protocol. In this case, PAP-2b was expressed with the additional 21 residues including the tag sequence.

Expression of PAP Isozymes—pREP9 harboring PAP isozymes (2 μg/35-mm dish) was transfected to 293 cells as described previously (27). After 3 days, cells were homogenized in the lysis buffer (27), and

membrane fractions were prepared for assaying various phosphatase activities.

Posttranslational Processing of HA-tagged PAP Isozymes—293 cells (40–60% confluence) cultured on 4-well plates (Nunc) were transfected with the expression plasmids (1.5 μg/well) harboring PAP-2a-HA and PAP-2b-HA. After 2 days, cells were washed with methionine-free culture medium and pulse-labeled with [^{35}S]methionine(40 μCi/ml) for 15 min. The cells were then chased for up to 45 min in the medium supplemented with 2 mM methionine as described (32). The chased cells were lyzed by sonication in 150 μl/well of the lysis buffer containing 1% Triton X-100, 200 mM KCl, 5 μM each of leupeptin and pepstain, and 25 mM triethanolamine-acetic acid (pH 7.2). The lysates were pretreated for 20 min on ice with 10 μl of 20% (w/v) formalin-fixed *Staphylococcus aureus* (Sigma) and 2 μl of 10% bovine albumin. The pretreated lysates were centrifuged for 5 min at 12,000 × *g*, and the supernatants were incubated for 60 min with the anti-HA tag antibody (0.5 μg/ml). The immunoprecipitates prepared (27) were denatured by heating at 65 °C for 10 min in 20 μl of 10 mM Tris-HCl (pH 7.4) containing 1% Triton X-100. After denaturation, the samples were treated with *N*-glycanase (10 units) or sialidase (1 milliunit) with or without 1 milliunit of endo-β-galactosidase according to the supplier's protocol. The mixture also contained 1 mM *o*-phenanthroline as a protease inhibitor and was incubated for 6 h at 37 °C. After enzymatic treatments, the samples were analyzed by SDS-12.5% PAGE (37). The radioactive bands were visualized by a BAS 2000 image analyzer (Fuji).

Analysis of PAP mRNAs in Stimulated HeLa Cells—Confluent HeLa cells in 35-mm dishes were starved for 40 h in the serum-free medium containing 0.1% bovine serum albumin. The cells were then cultured in the presence of 0.5 mM H_2O_2 or EGF(50 ng/ml) for up to 24 h. Total RNA was prepared from the treated cells as described previously (31). Reverse transcriptase-PCR amplification of the PAP mRNAs was done using Superscript preamplification system (Life Technologies, Inc.) following the manufacturer's protocol. The single-stranded cDNA primed by oligo(dT) was obtained from 2 μg of total RNA (31). The following PCR used 5 μM each of the specific primer sets: HPa3 (nucleotides 1–25) and HPa4 (5′-ATAGTGGCTATGTAGTTATTCCTG-3′, nucleotides 340–317) for PAP-2a; and HPb3 (1–24) and HPb4 (CACAGAGCA-CAGCGTCATTTATTG, 274–251) for PAP-2b amplification. The amplification using glyceraldehyde-3-phosphate dehydrogenase control amplimer sets (CLONTECH) was also done as a control. Amplification reaction was done on a GeneAmp PCR System 2400 (Perkin-Elmer Corp.) at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 2 min for the cycles that were preliminarily determined to yield linear amplification of PAP mRNAs (31): 22 cycles for PAP-2a, 23 cycles for PAP-2b, and 14 cycles for glyceraldehyde-3-phosphate dehydrogenase. The aliquots of the PCR products were resolved by 1.8% agarose gel electrophoresis and analyzed by Southern blotting as described previously (31). The probes were obtained by PCR amplification as described above using the cDNAs of PAP-2a and PAP-2b in pBluescript and labeled with ^{32}P by random priming.

Northern Hybridization—The human multiple tissue Northern blot (CLONTECH) was used according to the supplier's protocol. The labeled probes were the same as described for reverse transcriptase-PCR.

RESULTS

cDNA Cloning of Two Human PAP Isozymes—As already noted in our previous paper (27), the mouse 35-kDa PAP sequence gave, when searched for GenBank™ data base, several homologous sequences such as *Caenorhabditis elegans* T28D9.3p and *Saccharomyces cerevisiae* D9719.9p. Although these homologous proteins had no assigned functions, the alignment of the deduced amino acid sequences disclosed several conserved regions sharing 26–60% identical sequences with the mouse PAP. We additionally noted that a part of the mouse PAP sequence was significantly similar to the amino acid clusters conserved among bacterial nonspecific acid phosphatases (Ref. 28 and under "Discussion"). Based on these findings, we selected the amino acid sequence of Cys-153–Gln-234 of the mouse PAP (27) as a query sequence and repeated the homology search using TBLASTN program, resulting in the detection of several human cDNA fragments encoding similar amino acid sequences. These partial cDNAs could be aligned to yield two independent sequences (not shown). The first sequence was 626 bp long and encoded the C-terminal 161 residues. Since the encoded sequence was 81% identical to the corresponding region of the mouse PAP sequenced first (27), we designated the gene product PAP-2a. The second cDNA was 419 bp long and encoded the C-terminal 89 residues, which were only 38% identical to those of the mouse PAP. We thus designated the putative protein PAP-2b.

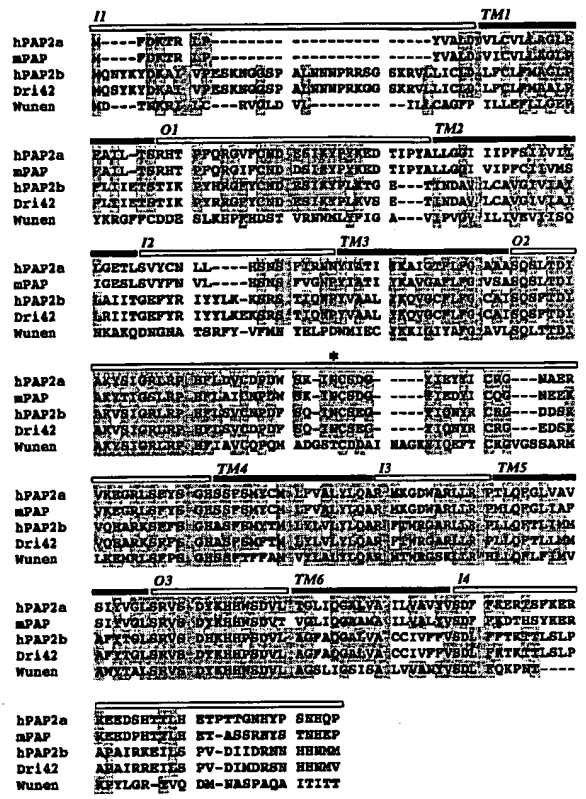

FIG. 1. **Sequence comparison of the type 2 PAP isozymes.** The amino acid sequences of two human PAP isozymes, hPAP2a and hPAP2b, are aligned with those of mouse PAP (27), rat Dri42(30), and *Drosophila* Wunen (29). *Dashes* indicate gaps inserted to maximize alignment, and identical amino acids are *shaded*. The *asterisk* indicates the *N*-linked glycosylation site conserved in all of the mammalian enzymes. The transmembrane segments of the mammalian PAPs are predicted by PHDhtm program of The PredictProtein server at The European Molecular Biology Laboratory. The transmembrane disposition of the mammalian PAPs is tentatively assigned based on the results obtained for rat Dri42 (30). The intracellular (*I*), transmembrane (*TM*), and extracellular (*O*) segments are numbered from the N terminus as indicated in the figure. The nucleotide sequences of hPAP-2a and hPAP-2b (not shown) are listed in the data base under the accession numbers AB000888 and AB000889, respectively.

To obtain the full-length cDNAs containing the complete coding regions for the putative PAP-2a and -2b, we performed the two-step amplification of cDNAs from HepG2 RNA as described under "Experimental Procedures." The amplified cDNAs encoding PAP-2a and -2b were 937 and 1024 bp long, respectively (not shown), and contained the open reading frames at nucleotides 48–902 and 7–942, respectively. In both cDNAs, we recognized neither in-frame stop codons in the 5′-noncoding regions nor polyadenylation sites in the 3′-flanking regions. We thus could not deny the possibility that the cDNAs obtained did not cover the whole coding regions for the two putative PAPs. However, the repeated 5′-RACE procedures failed to further extend the cDNAs, and we judged from the similarities of the two sequences that the cDNAs we obtained contained the full-length coding regions as described below.

As shown in Fig. 1, PAP-2a and -2b consisted of 284 and 311 amino acid residues with the calculated M_r of 32,158 and

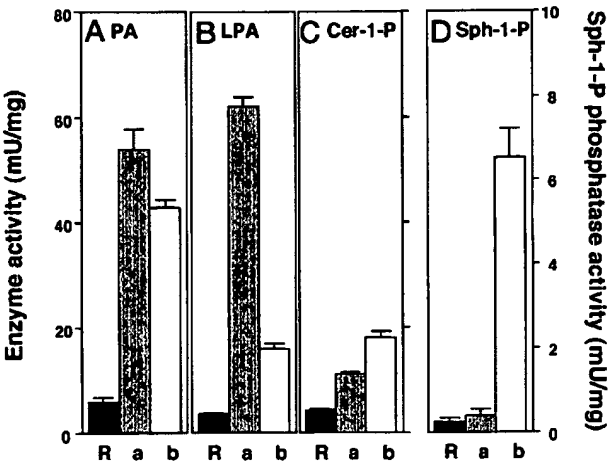

FIG. 2. **Lipid phosphate phosphatase activities of the expressed PAP isozymes.** The cDNAs encoding PAP-2a and -2b were subcloned into pREP9 expression vector and transfected into 293 cells. After 3 days, membranes were obtained from cells transfected with the vector alone (*R*), PAP-2a (*a*), and PAP-2b (*b*). The membranes (<10 μg of protein) were subjected to the enzyme assay using as substrates PA (*A*), lyso-PA (*LPA*) (*B*), ceramide-1-phophosphate (*Cer-1-P*) (*C*), and sphingosine-1-phosphate (*Sph-1-P*) (*D*) as described under "Experimental Procedures." The results are means ± S.D. of three repeated experiments.

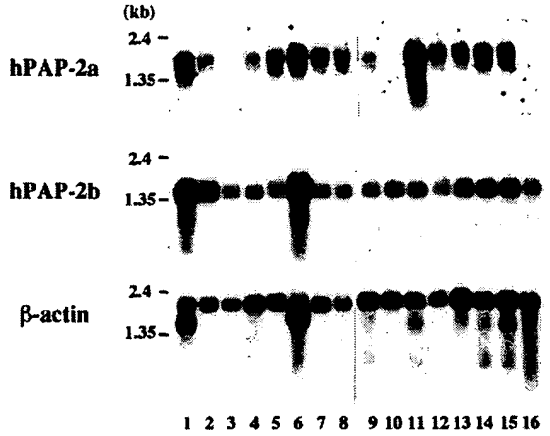

FIG. 3. **Northern hybridization analysis of the PAP isozymes.** The multiple human tissue Northern blots (CLONTECH) were hybridized with ^{32}P-labeled probes specific to each PAP and the control actin probe as described under "Experimental Procedures." *1*, heart; *2*, brain; *3*, placenta; *4*, lung; *5*, liver; *6*, skeletal muscle; *7*, kidney; *8*, pancreas; *9*, spleen; *10*, thymus; *11*, prostate; *12*, testis; *13*, ovary; *14*, small intestine; *15*, colon; *16*, peripheral blood leukocytes. *hPAP*, human PAP; kb, kilobases.

35,119, respectively. The sequences of the two putative PAPs are similar to but distinctly different from each other, sharing only 47% identical sequence. PAP-2a and the mouse PAP previously sequenced are highly similar to each other, sharing overall 83% identical sequence, thus indicating that PAP-2a is a human homolog of the mouse PAP. In this context we noted that the N-terminal 25 residues of PAP-2a are the same as have been determined chemically for the purified porcine thymus 35-kDa PAP (27). Unexpectedly, PAP-2b shares 94% identical sequence with rat Dri42 protein (30). Dri42 has been cloned as the gene product up-regulated during the epithelial differentiation in rat small intestine (30). We thus cloned a human counterpart of rat Dri42 in an attempt to characterize PAP isozymes. In addition, *Drosophila* Wunen protein, which was discovered as a repulsive gene product responsible for regulating the germ cell migration in the embryo (29), shares 39 and 34% identical sequences with PAP-2a and PAP-2b, respectively. The four mammalian PAP homologs contained a single conserved *N*-glycosylation site, and PAP-2a and -2b were indeed confirmed to be glycoproteins as will be described later. These proteins contained six transmembrane regions as revealed by the hydropathy plot (not shown). Since the transmembrane disposition of the rat Dri42 protein was characterized in detail (30), the sequences of these PAPs could be tentatively classified into intracellular, transmembrane, and extracellular regions as depicted in Fig. 1.

Enzymic Properties of the Expressed PAP Isozymes—To confirm that the two cDNAs encoded the PAP enzymes, the cDNAs were subcloned into the expression vector, pREP9, and transfected to 293 cells. The membrane fractions obtained 3 days after transfection were subjected to the enzyme assay as described under "Experimental Procedures." The membranes prepared from cells transfected with the PAP-2a and -2b cDNAs showed 9- and 7-fold higher PAP activities, respectively, when compared with the cells transfected with the control vector alone (Fig. 2, *panel A*). In this case no changes of the enzyme activity were detected for the cytosolic fractions. We also confirmed that both PAP-2a and -2b activities were inhibited by propranolol and sphingosine, very similarly as described for the purified porcine thymus PAP (23). Since both PAP-2a and -2b activities were insensitive to *N*-ethylmaleimide and independent of Mg^{2+} (not shown), the two PAP isozymes were suggested to be the type 2, plasma membrane-bound enzymes (9).

Waggoner *et al.* (17) recently reported that the type 2 PAP purified from rat liver dephosphorylates lyso-PA, ceramide-1-phosphate, and sphingosine-1-phosphate in addition to PA. Furthermore, the same enzyme preparation was described as capable of dephosphorylating DG pyrophosphate (21). In view of such a broad substrate specificity described for the type 2 PAP, we tested various substrates for the two PAP isozymes expressed in 293 cells (Fig. 2). The activity of PAP-2a to lyso-PA was particularly high (15-fold increase from the control), and a significant increase of ceramide-1-phosphate-hydrolyzing activity (2.5-fold increase) was also observed for this isozyme. In contrast, the transfection of PAP-2a failed to increase the activity toward sphingosine-1-phosphate (Fig. 2, *panel D*). The transfection of the PAP-2b cDNA, on the other hand, increased the activities to lyso-PA, ceramide-1-phosphate, and sphingosine-1-phosphate by 4-, 4-, and 26-fold over the control membranes. In these experiments, various lipid phosphate phosphatase activities were variably increased due to the different levels of the endogenous activities. All enzyme assays were done simply under the fixed reaction conditions, which gave maximum activities. Although detailed kinetic analysis is needed, it is clear that the substrate specificities of the two PAPs cloned are distinct from each other and that dephosphorylation of sphingosine-1-phosphate by PAP-2a occurs to a negligible extent, if any. In this respect, the properties of PAP-2b are more similar to those of rat liver enzyme(17), which degrades all of these substrates at similar rates.

The Expression Patterns of the PAP Isozymes—We first studied the tissue-dependent expression of the two PAPs using specific probes and the commercial multiple tissue Northern blot human blots. The sizes of mRNAs of PAP-2a and -2b were estimated to be 1.74 and 1.4 kb, respectively (Fig. 3). We noted clearly distinct distribution patterns of the two mRNAs. The PAP-2b mRNA was detected in all tissues examined, indicating a ubiquitous expression of this PAP isozyme. PAP-2a expression, on the other hand, was markedly variable, depending on the tissues, being highly abundant in the prostate but undetectable in the thymus, placenta, and leukocytes.

We previously showed that the mouse 35-kDa PAP, a homolog of PAP-2a, was highly similar to the *hic*53 gene product

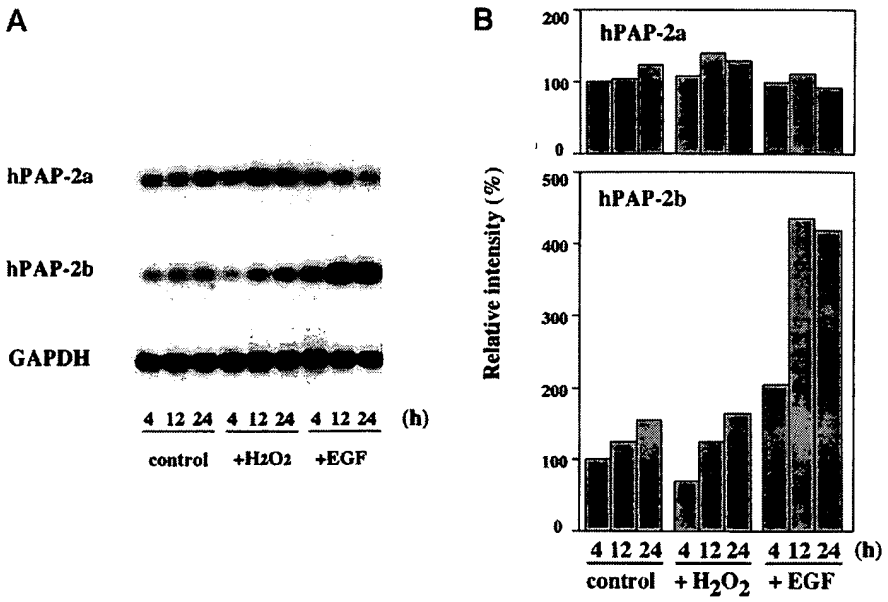

FIG. 4. **Reverse transcriptase-PCR analysis of the PAP expression in HeLa cells.** *A*, quiescent HeLa cells were exposed for different periods as indicated to 0.5 mM H_2O_2 or 50 ng/ml epidermal growth factor. The total cellular RNA (2 μg) was subjected to reverse transcriptase-PCR in which 22, 23, and 14 cycles of PCR amplifications were done for PAP-2a, -2b, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), respectively. The PCR products were detected by Southern blotting using radiolabeled probes as described under "Experimental Procedures." The results are representative of twice-repeated experiments. *hPAP*, human PAP. *B*, relative signal intensities normalized with glyceraldehyde-3-phosphate dehydrogenase signals as determined by densitometric scanning of the autoradiograms.

described to be induced upon H_2O_2 treatment of mouse osteoblastic cell line (38). We were thus interested to see if the expression of the two PAPs was affected in cells treated with various agonists, including H_2O_2. For this purpose, quiescent HeLa cells were exposed to H_2O_2, and the time-dependent changes of the levels of PAP mRNAs were estimated by reverse transcriptase-PCR as shown in Fig. 4. We found that the expression of both PAPs was not affected significantly by the treatment with H_2O_2. Such negative results have been obtained using different concentrations of H_2O_2 (0–5 mM) and using other cells like HepG2 and PC3 (not shown). Similarly, the expression of the two PAPs was not affected by a variety of other agonists, including tumor necrosis factor α (at 50 ng/ml), tumor growth factor β1 (2.5 ng/ml), phorbol myristate acetate (200 nM), and calcium ionophore A23817(100 nM) (not shown). However, EGF caused an exceptional sustained increase (up to 3-fold) of the mRNA of PAP-2b but not of PAP-2a as shown in Fig. 4. In these experiments we observed a gradual increase of the PAP-2b mRNA during the control incubations that was reproducible. The reason for this phenomenon remains unknown. The results of Northern blotting and reverse transcriptase-PCR analysis of cellular mRNAs as described above suggest that the levels of expression of PAP-2a and 2b are regulated by distinct mechanisms.

Posttranslational Processing of the PAP Isozymes—The mouse 35-kDa PAP has already been shown to be a glycoprotein and possess two consensus *N*-linked glycosylation sites (27). The alignment of sequences of four mammalian PAP homologs achieved in the present study (Fig. 1) showed that these enzymes have a single (instead of two) *N*-glycosylation site conserved. It is thus quite likely that PAP-2a and -2b are glycosylated at Asn-142 and Asn-170, respectively. To assess the posttranslational processing of the two PAPs, 293 cells transfected with HA-tagged enzymes were pulse-labeled with [^{35}S]methionine, and anti HA epitope tag immunoprecipitates obtained from the chased cells were analyzed by SDS-PAGE (Fig. 5). In these experiments, calculated molecular masses of epitope-tagged PAP-2a and -2b were estimated to be approximately 35 and 38 kDa, respectively. In the case of PAP-2a (Fig. 5*A*), the labeled cells contained a major 41- and a minor 37-kDa bands before chase. During the chase periods, the 41-kDa band gradually decreased and was replaced with a diffuse 45-kDa species. When treated with *N*-glycanase, both 41- and 45-kDa

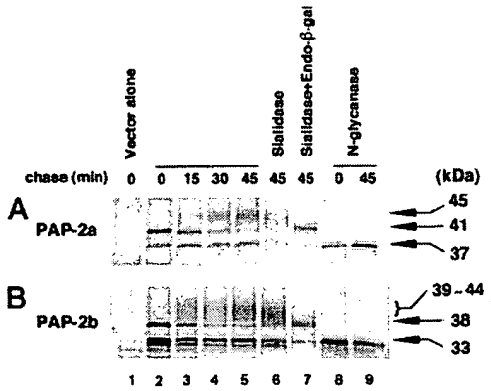

FIG. 5. **Posttranslational processing of HA-tagged PAP isozymes.** 293 cells were transfected with the expression plasmids harboring PAP-2a-HA and PAP-2b-HA. After 2 days, cells were pulse-labeled with [^{35}S]methionine for 15 min followed by chase for the indicated periods. HA-tagged PAP isozymes were immunoprecipitated from cell lysates using anti-HA epitope tag antibody. Aliquots of undigested immunoprecipitates (*lanes 2–5*), sialidase-digested (*lane 6*), sialidase/endo-β-galactosidase (*Endo-β-gal*)-digested (*lane 7*), or *N*-glycanase-digested (*lanes 8* and *9*) were analyzed by SDS-12.5% PAGE. Immunoprecipitates prepared from cells transfected with the vector alone were also analyzed as a control (*lane 1*).

bands shifted to 37 kDa. The migration of the 45-kDa diffuse band was slightly affected by sialidase treatment (Fig. 5*A*, *lane 6*), and this band became 41 kDa when treated further with endo-β-galactosidase. The sensitivity to endo-β-galactosidase strongly suggests the presence of polylactosaminoglycans (39). These findings indicate that the 37-kDa band is a nonglycosylated form and that the 41-kDa species contains a high mannose-type oligosaccharide. The diffuse 45-kDa should represent a mature form of PAP-2a containing an *N*-linked oligosaccharide modified by the addition of polylactosaminoglycan and sialic acids (39). The same rationale could be applied to PAP-2b (Fig. 5*B*). In this case, the 33-kDa band represents a nonglycosylated enzyme, and the 38-kDa species contains an *N*-linked high mannose-type sugar chain. The diffuse bands at 39–44 kDa should be the mature forms of PAP-2b containing polylactosaminoglycans. It was noted that the oligosaccharide chain of PAP-2b was more heterogeneous than that of PAP-2a, as shown by the presence of at least 4 bands at 39–44 kDa(Fig.

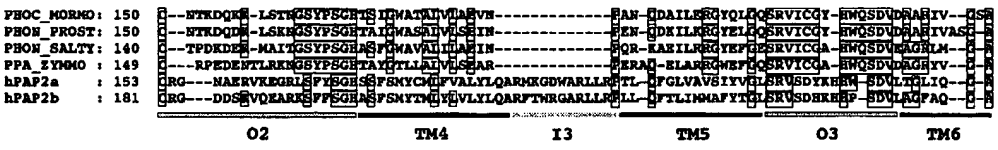

FIG. 6. **Sequence comparison of the PAP isozymes and class A bacterial acid phosphatases.** The PAP isozymes show a significant similarity to cluster 3292.5 (CRSeq data base), which is the most conserved region of class A bacterial acid phosphatase and apyrase. Identical amino acids conserved in the two gene families are *boxed*. The putative structural segments assigned to PAP isozymes are indicated as given in Fig. 1. Accession numbers for *Morganella morganii* major phosphate-irrepressible acid phosphatase (*PHOC MORMO*), *Providencia stuartii*-nonspecific acid phosphatase (*PHON PROST*), *Salmonella typhimurium*-nonspecific acid phosphatase (*PHON SALTY*), and *Zymomonas mobilis* acid phosphatase (*PPA ZYMMO*) are P28581, P26975, P26976, and P14924, respectively. *I*, intracellular; *TM*, transmembrane; and *O*, extracellular.

5*B*, *lane 5*). In these experiments, the migration of the HA-tagged enzymes upon SDS-PAGE was somewhat deviated from their expected molecular masses for unknown reasons.

DISCUSSION

We have cloned two human PAP isozymes, both of which can be classified as the type 2 enzymes. One isozyme, designated PAP-2b, was judged to be the human homolog of rat Dri42, sharing 94% identical sequence. The other isozyme, PAP-2a, was considered to be the human counterpart of the mouse 35 kDa PAP (27), based on the sequence comparison. Rat Dri42 has been cloned as a gene induced upon epithelial differentiation in the small intestine (30). It was also noted that the two human PAPs exhibit a significant similarity to *Drosophila* Wunen, which regulates the germ cell migration (29). The sequencing of the mammalian PAPs thus unexpectedly disclosed important roles of the type 2 PAPs in basic biological functions such as cell migration, differentiation, and growth. Since the Dri42 protein is now confirmed to possess the PAP enzyme activity, it is highly likely that the Wunen also exerts its regulatory functions by catalyzing the dephosphorylation of PA and/or other lipid phosphates. One of the unresolved questions concerns the intracellular localization of Dri42 and PAP-2b. Rat Dri42 was concluded to reside in the endoplasmic reticulum, based on the results of immunostaining of the endogenous protein in the small intestine and of the overexpressed fusion protein with chloramphenicol acetyltransferase (30). Furthermore, the fusion protein obtained from the pulse-chased cells did not acquire Golgi modification of its *N*-linked oligosaccharide (30). In the case of PAP-2b, the sensitivity of its oligosaccharide chain to endo-β-galactosidase (Fig. 5) at least indicates its post-Golgi localization (39). In the experiments not shown, the intracellular distribution of both PAP-2a and -2b expressed in 293 cells as GFP-fusion proteins was distinct from that of the simultaneously stained calnexin, which is known to serve as a marker for the endoplasmic reticulum (40). The results of the parallel experiments using PAP-2a, the mouse and porcine homologs of which have been confirmed to be plasma membrane-bound (27), also support the plasma membrane localization of these enzymes. We thus do not know why the two almost identical proteins are different in their intracellular localization.

Although we failed to account for the discrepant localization of PAP-2b and Dri42, the transmembrane disposition of Dri42 characterized in detail (30) provides useful information on the shape and function of the mammalian type 2 PAPs. As has been depicted in Fig. 1, the mammalian PAP can now be proposed to contain four intracellular segments, including both N- and C-terminal regions, six transmembrane regions, and three extracellular loops. Such a transmembrane disposition assigned to these molecules is reminiscent of channel proteins rather than lipid phosphatases, as has been discussed for Dri42. However, a novel phosphatase motif has been proposed during the preparation of this manuscript (41, 42). It has been proposed that there exists a novel phosphatase superfamily possessing a common catalytic mechanism and consisting of the mouse 35-kDa PAP, haloperoxidases, acid phosphatases, glucose-6-phosphatase, and many others (41, 42). Although further work is needed to substantiate the proposed catalytic mechanism, it is clear that the type 2 PAP represents a novel class of phosphatases. Although not tested in the present study, the dephosphorylation of DG pyrophosphate appears to be a common property of various type 2 PAPs, as has been shown for rat liver enzyme (21) and *E. coli pgp*B gene product (43). Identification of amino acid residues essential for the PAP activity is urgently needed to define the catalytic mechanisms of these novel phosphatases. In this context, we noted that a part of the sequences of the type 2 PAPs is significantly conserved in the amino acid clusters found in bacterial acid phosphatase family (Fig. 6). These acid phosphatases are soluble proteins with approximately 250 amino acid residues. Since the acid phosphatases have no other conserved sequences than the amino acid clusters given in Fig. 6, these clusters are quite likely included in their catalytic sites. Although similar findings have already been reported (41, 42), we intend to point out the fact that the majority of the conserved residues reside in the second and third extracellular regions presently assigned to the mammalian PAPs. We therefore cannot rule out the possibility that the type 2 PAPs so far cloned may dephosphorylate extracellular substrates acting as ectoenzymes. Indeed, there are several reports describing ecto-PAP, whose properties are strikingly similar to those of the type 2 PAPs(44, 45). This intriguing possibility awaits further investigation.

The type 2 PAP purified from rat liver has been shown to posses a broad substrate specificity dephosphorylating a variety of lipid phosphates, including DG pyrophosphate and those derived from sphingolipids (17, 21). The two PAPs cloned in the present work were also confirmed to have broad substrate specificity, although the ability to degrade sphingosine-1-phosphate could be detected only for PAP-2b. The rat liver enzyme was described to be a 51–53-kDa protein, which became 30 kDa when treated with *N*-glycanase (25). The size of the glycosylated liver enzyme does not match those so far estimated for the sequenced PAPs. This may indicate the presence of the third PAP isozyme. However, the fact that we detected only two classes of homologous sequences in the data base searches appears to suggest that there exist only two PAP isozymes in mammalian tissues. If this is the case, the activity to sphingosine-1-phosphate suggests that PAP-2b may be the human counterpart of rat liver enzyme despite the apparent differences in their molecular sizes.

The expression pattern of PAP-2a among human tissues was much more variable when compared with that of PAP-2b, which was expressed relatively ubiquitously. Despite the close relationship to mouse *hic* 53 clone previously described (27), the cellular PAP-2a failed to be induced under a variety of experimental conditions including the treatment with H_2O_2.

This discrepancy may be due to the use of different cells and animal species. In contrast, the cellular PAP-2b was significantly induced by EGF. In view of the up-regulation of rat Dri42 during epithelial differentiation, the expression of PAP-2b appears to be influenced by extracellular signals. The tissue-dependent modes of the PAP-2a expression may suggest its involvement in as yet undefined tissue-specifc functions.

The type 2 PAP has attracted attention as the enzyme participating in the control of signal transduction mediated by phospholipase D (5). The knowledge on the molecular structure of this enzyme led to the discovery of its unexpected functions in basic biological functions. Further studies on this group of PAP and on the type 1 cytosolic PAP, whose structure remains unknown, would reveal their potentially diverse functions in lipid metabolism and signal transduction.

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Human Type 2 Phosphatidic Acid Phosphohydrolases

SUBSTRATE SPECIFICITY OF THE TYPE 2a, 2b, AND 2c ENZYMES AND CELL SURFACE ACTIVITY OF THE 2a ISOFORM*

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Phosphatidic acid (PA), lysophosphatidic acid, ceramide 1-phosphate (C1P), and sphingosine 1-phosphate (S1P) are lipid mediators generated by phospholipases, sphingomyelinases, and lipid kinases. The major pathway for degradation of these lipids is dephosphorylation catalyzed by members of two classes (types 1 and 2) of phosphohydrolase activities (PAPs). cDNAs encoding two type 2 PAPs, PAP-2a and -2b, have been expressed by transient transfection and shown to catalyze hydrolysis of PA, C1P, and S1P (Kai, M., Wada, I., Imai, S., Sakane, F. and Kanoh, H. (1997) *J. Biol. Chem.* 272, 24572–24578). We report the cloning and expression of a third type 2 PAP enzyme (288 amino acids, predicted molecular mass of 32.6 kDa), PAP-2c, which exhibits 54 and 43% sequence homology to PAPs 2a and 2b. Expression of HA epitope-tagged PAP-2a, -2b, and 2c in HEK293 cells produced immunoreactive proteins and increased membrane-associated PAP activity. Sf9 insect cells contain very low endogenous PAP activity. Recombinant expression of the three PAP enzymes using baculovirus vectors produces dramatic increases in membrane-associated Mg^{2+}-independent, *N*-ethylmaleimide-insensitive PAP activity. Expression of PAP-2a but not PAP-2b or -2c resulted in high levels of cell surface PAP activity in intact insect cells. Kinetic analysis of PAP-2a, -2b, and -2c activity against PA, lysophosphatidic acid, C1P, and S1P presented in mixed micelles of Triton X-100 revealed differences in substrate specificity and susceptibility to inhibition by sphingosine, Zn^{2+}, and propranol.

Phosphatidate phosphohydrolase (PAP)[1] catalyzes the dephosphorylation of phosphatidic acid (PA) to form diacylglycerol (DG). This enzyme was first recognized as a pivotal component of metabolic pathways controlling the synthesis of glycerophospholipids and triacylglycerols (1). More recent advances have focused attention on PA as a receptor-generated intracellular mediator formed by the phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine (PC). PA generated in this manner appears to act on a variety of cell-specific target proteins that include atypical protein kinase C isoforms, the Raf protein kinases, the Ras GTPase-activating protein, several proteins involved in cytoskeletal organization, and the neutrophil respiratory burst oxidase. In this case, PAP-catalyzed hydrolysis of PA serves to terminate the signaling functions of PA and concurrently generates diacylglycerol for activation of conventional Ca^{2+} and phospholipid-dependent protein kinase C enzymes. DG kinase generates PA by ATP-dependent phosphorylation of DG. Presumably the subcellular localization, catalytic, and regulatory properties of these different lipid-metabolizing enzymes will dictate the source, fate, and signaling functions of PA and DG (2, 3).

PAP activity is widely expressed, and two classes of these enzymes can be distinguished in mammalian cells on the basis of their subcellular distribution and catalytic properties (4). Type 1 activity (PAP-1) is associated with the cytosol and endoplasmic reticulum and appears to redistribute from the soluble to membrane compartment upon treatment of hepatocytes with glucagon and glucocorticoids (4, 5). PAP-1 activity is sensitive to inhibition by sulfhydryl reagents (most notably *N*-ethylmaleimide (NEM)) and displays an absolute requirement for Mg^{2+}. PAP-2 activity has been localized to the plasma membrane and, in contrast to PAP-1, is independent of Mg^{2+} and insensitive to inhibition by NEM (4–8). PAP-2 activity is presumably directed toward the inner leaflet of the plasma membrane, although several reports also describe a cell surface enzyme with PAP-2-like properties that may serve to terminate the receptor-directed signaling functions of lyso-PA (LPA) and related compounds (9, 10). Sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P) phosphatase activities have also been described in mammalian cells, and purified preparations of rat liver PAP-2 also catalyze the hydrolysis of these lipid phosphomonoesters (11). C1P, S1P, and their hydrolysis products exhibit a number of interesting biological activities and may function as intra- and possibly extracellular signaling molecules (12). The inference drawn from these studies is that PAP-1 most likely functions in lipid synthesis, while PAP-2 may have an important role to play in modulating the signaling functions of PA and LPA as well as lipid phosphomonoesters derived from sphingomyelin.

The tight association of PAP with membranes and the low abundance of the proteins has hampered purification attempts. PAP-1 has not yet been isolated. Several groups have prepared highly enriched preparations of PAP-2 from a number of tissue sources (6–8). Rat liver PAP-2 was identified as a glycosylated protein of about 50 kDa that could be converted to a 28-kDa protein by *N*-glycanase treatment (6). A mouse cDNA (mPAP-2a) encoding a protein that is highly similar to a heat-inducible gene product (Hic53) has recently been isolated. When ex-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF017116, AF017786, AF047760 (human PAP-2a, -2b, and -2c, respectively).

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[1] The abbreviations used are: PAP, phosphatidic acid phosphohydrolase; PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; LPA, lysophosphatidic acid; C1P, ceramide 1-phosphate; S1P, sphingosine 1-phosphate; NEM, *N*-ethylmaleimide; DG, diacylglycerol; HA, hemagglutinin; EST, expressed sequence tag; hPAP, human PAP.

pressed in HEK 293 cells, this protein localizes to the plasma membrane and is accompanied by a Mg^{2+}-independent PAP activity (13). A human cDNA encoding a second mammalian PAP-2 enzyme, hPAP-2b (the human homolog of a previously described rat intestinal epithelial cell endoplasmic reticulum-resident protein, Dri42), has been cloned (14, 15). Transient expression of PAP-2a and -2b in HEK-293 cells produced increases in membrane-associated PA and C1P phosphatase activities. Hydrophobicity analysis suggests that PAP-2a and -2b are integral membrane proteins with six hydrophobic membrane-spanning regions (13–16). Comparison of the sequence of mPAP-2a with other phosphatases defines a protein motif composed of three regions of conserved sequence. PAP-2 homologs have been identified in yeast and *Drosophila* (16–20).

We sought to identify further human PAP homologs, and a search of expressed sequence tag data bases identified a number of candidate sequences. Full-length cDNAs encoding three of these (PAP-2a, PAP-2b, and a novel PAP-2 isoform, PAP-2c) have been cloned. These enzymes have been expressed as HA epitope-tagged proteins by transient transfection of HEK-293 cells and in Sf9 insect cells using baculovirus vectors. These systems have been used to investigate the cell surface activity of the three PAP enzymes, to examine their specificity for lipid phosphomonoester substrates presented as components of non-ionic detergent micelles and susceptibilities to inhibition by a number of agents that have been widely used as modulators of PAP-2 activity in intact and broken cell systems.

EXPERIMENTAL PROCEDURES

Isolation and Analysis of hPAP-2a, -2b, and -2c cDNAs—We conducted blast searches of the GenBank™ data base of expressed sequence tags (ESTs) using regions of sequence conserved among mouse PAP-2 and a variety of previously recognized homologs including the product of the *Drosophila wunen* gene and four *Saccharomyces cerevisiae* open reading frames. We identified a number of candidate human sequences in this manner. Sequences encoding human homologs of mouse PAP-2a and rat Dri42 and a third apparently novel sequence were selected for further study. These cDNAs and the proteins they encode are hereafter referred to as PAP-2a, -2b, and -2c. An open reading frame containing the complete cDNA sequence of hPAP-2a was constructed from overlapping ESTs, and a complete cDNA was amplified from reverse-transcribed HL-60 cell cDNA by polymerase chain reaction using primers with the sequences 5′-GCTCTAGAACCATGTTTGACAAGACGCGG-3′ (forward) and 5′-CAGCCCGGGCTCGGCACCCTGCTG-3′ (reverse) using standard methodology (21). The cDNA was ligated into pGEM-7ZF. One of the ESTs identified (accession number U79294) appeared to contain a complete open reading frame encoding hPAP-2b. This EST was obtained from the IMAGE Consortium, and the hPAP-2b cDNA was sequenced by a combination of manual sequencing using Sequenase 2.0 and automated sequencing performed in the center for analysis of macromolecules at Stony Brook. An open reading frame containing the complete cDNA sequence of hPAP-2c was constructed from overlapping ESTs, and a complete cDNA was amplified from reverse-transcribed HeLa cDNA using primers with the sequences 5′-GGTTCTAGAACCATGCAGCGGAGGTGGGTC-3′ (forward) and 5′-GGGGGATCCCCTCAGGAGGAGGAGTGCGGG-3′ (reverse) using standard methodologies (21). The cDNA was ligated into pGEM7ZF and sequenced by a combination of manual sequencing using Sequenase 2.0 and automated sequencing performed in the center for analysis of macromolecules at Stony Brook.

Transient Expression of hPAP-2a, -2b, and -2c in HEK-293 Cells—hPAP-2a, -2b, and -2c cDNAs were subcloned into pCGN, which is a cytomegalovirus-based vector for expression of proteins with an NH_2-terminal HA epitope tag. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 35-mm diameter lysine-coated dishes of 50% confluent cells were transfected with 1 μg of pCGN hPAP-2a, pCGN hPAP-2b, or pCGN hPAP-2c using lipofectamine in Opti-MEM (Life Technologies, Inc.). The transfection medium was removed after 24 h and replaced with complete Dulbecco's modified Eagle's medium. The cells were harvested 24 h later by washing in phosphate-buffered saline followed by scraping into ice-cold lysis buffer containing 20 mM Tris, pH 7.5, 5 mM EGTA, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride. The lysate was disrupted by sonication on ice with a probe-type sonicator, and the material was used in assays within 24–48 h as described below. In some cases, the lysate obtained was fractionated into total membrane and cytosolic fractions by centrifugation at 30,000 × *g* at 4 °C. The membrane fraction was resuspended in ice-cold lysis buffer by vortexing.

Baculovirus Expression of PAP-2a, -2b, and -2c—The PAP-2a, -2b, and -2c cDNAs were subcloned into pFastBac (Life Technologies, Inc.), and recombinant baculoviruses were generated by sequential transformation of DH10Bac cells, isolation of recombinant bacmids, and transfection of Sf9 cells using Cellfectin reagent. Recombinant baculoviruses were selected and propagated using standard procedures to generate high titer stocks (22). Monolayers of exponentially growing insect cells cultured in complete Grace's medium containing antibiotics, antimycotics, 10% fetal bovine serum and supplemented with lactalbumin and Yeastolate (generally 20 × 10^6 cells in a 225-cm^2 flask) were infected with recombinant baculoviruses for expression of the PAP enzymes or of a control protein (PLD2) at a multiplicity of 10. The cells were cultured for 48 h at 27 °C. For assays using intact insect cells, the monolayer of cells was carefully washed with unsupplemented Grace's medium, and the cells were dislodged by gentle pipetting and transferred to a 50-ml conical centrifuge tube. The intact cells were sedimented by centrifugation at 100 × *g* for 10 min at room temperature and resuspended in unsupplemented Grace's medium. The cells were kept at 27 °C and used within 6 h of isolation. Where indicated, for determinations of total lactate dehydrogenase or PAP activity, these cells were disrupted by sonication on ice (see below), and the sonicated material was kept on ice before use.

For studies using Sf9 cell membranes or detergent-extracted membrane proteins, the monolayers of infected cells were washed gently with phosphate-buffered saline and lysed by the addition of 4 ml of ice-cold lysis buffer and scraping. The cell suspension was transferred to a 15-ml conical tube, and the cells were disrupted by sonication (Vertis Systems Sonifier), 10 10-s pulses on ice. The disrupted cells were centrifuged at 20,000 × *g* at 4 °C for 20 min. The cytosolic fraction was removed, and the membrane fraction was resuspended in ice-cold lysis buffer. Detergent extracts were prepared from the membranes by the addition of Triton X-100 and β-D-octylglucopyranoside to final concentrations of 1% followed by incubation at 4 °C with constant rocking for 1 h. The solubilized material was centrifuged at 26,000 × *g* at 4 °C for 30 min, and the supernatant was removed.

Preparation of Substrates—[^{32}P]PA, C1P, and LPA were prepared by phosphorylation of DG, C8 ceramide, or monooleoylglycerol (Avanti Polar Lipids) using *Escherichia coli* DG kinase (Calbiochem) and [γ-^{32}P]ATP (ICN) (11). The reactions were terminated by extraction with acidified $CHCl_3$ and MeOH, and the dried organic phases obtained were resuspended in 0.4 ml of 20:9:1 $CHCl_3$/MeOH/H_2O (solvent A) and neutralized by the addition of a small volume of 20% NH_4OH in MeOH. This material was applied to an Econosil NH_2 5U high pressure liquid chromatography column (250 × 4.2 mm) (Alltech Associates). The column was washed with 20 ml of solvent A and then eluted with a 40-ml linear gradient of 0–1 M ammonium acetate in solvent A. 0.5-ml fractions of the eluant were collected, and associated radioactivity was determined by liquid scintillation counting. ^{32}P-Labeled products were pooled and extracted from the eluant by the addition of 3 M HCl and $CHCl_3$ to give two phases. [^{32}P]PA and [^{32}P]C1P prepared in this way were dried and resuspended in a small volume of solvent A. The lipids were stored at −20 °C until use. [^{32}P]S1P was prepared by acid hydrolysis of [^{32}P]C1P and purified by thin layer chromatography on silica gel plates (11) or by phosphorylation of sphingosine using Swiss 3T3 cells as a source of sphingosine kinase (23). Radiolabeled material was identified by autoradiography, the silica was scraped from the plate, and lipids were eluted by the addition of acidified $CHCl_3$/MeOH. The eluted material was stored at −20 °C. Dipalmitoyl PA was obtained from Avanti Polar Lipids. Unlabeled C8 C1P and S1P were obtained from Biomol Inc. Sphingosine was from Avanti Polar lipids.

PAP Assays—The assay procedures used were adapted from those described by others (6, 7). In brief, assays were performed in medium containing 20 mM Tris, pH 7.5, 1 mM EGTA, and 2 mM EDTA, and in some cases the $MgCl_2$ concentration of the assay medium was varied by the addition of $MgCl_2$ as indicated. C1P and S1P were stored as 1 mg/ml solutions in 1 mM Triton X-100. ^{32}P-Labeled lipid substrates were dried under vacuum and resuspended in 6.4 mM Triton X-100. Unlabeled PA was mixed with the radiolabeled substrate before drying and resuspension, while C1P and S1P were added to appropriate dried radiolabeled substrates from stock solutions in Triton X-100. In some experiments, dodecyl β-D-maltoside was substituted for Triton X-100. The substrates were dispersed by bath sonication and vortexing. Unless otherwise noted, the assay volume was 100 μl, and each assay contained final

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                                                     I
            1            15 16           30 31           45 46           60 61           75 76           90
concensus                                     R++ ++LD++C++ +AGLPF+I+ +     P  RG+ CND+ SIKYP K  +TI  +
hPAP-2a     --------------- ------------MFD KTRLPYVALDVLCVL LAGLPFAIFTSRHIT SRHTPFQRGVFCNDE SIKYPYKE-DTIPYA
hPAP-2b     MQNYKYDKAIVPESK NGGSPALNNNPRRSG SKRVLLICLDLFCLF MAGLPFLIIETST-- --IKPYHRGFYCNDE SIKYPLKTGETINDA
hpap-2c     --------------- --------------M QRRWVFVLLDVLCLL VASLPFAILTLVN-- ---APYKRGFYCGDD SIRYPYRP-DTITHG

                   II                                        III                            A
            91          105 106         120 121         135 136         150 151         165 166      *  180
concensus   ++++++I++ +++++  GE   +Y     S  S I N Y+A +YK + G FLFG+A+SQS+TD IAK  IGRLRP FL  VC PD+S +NCS+
hPAP-2a     LLGGIIIPFSIIVII LGETLSVYCNLLHSN SFIRNNYIATIYKAI GTFLFGAAASQSLTD IAKYSIGRLRPHFLD VCDPDWSKINCSDGY
hPAP-2b     VLCAVGIVIAILAII TGEFYRIYYLKK-SR STIQNPYVAALYKQV GCFLFGCAISQSFTD IAKVSIGRLRPHFLS VCNPDFSQINCSEGY
hPAP-2c     LMAGVTITATVILVS AGEAYLVYTDRLYSR SDFNN-YVAAVYKVL GTFLFGAAVSQSLTD LAKYMIGRLRPNFLA VCDPDWSRVNCSVYV

                                       B           IV                          V               C
            181         195 196         210 211         225 226         240 241         255 256         270
concensus      Y CRG    V E +R SF SGH SF MY  ML ++LY+QAR  +  +ARLLRP LQF L+ ++ +Y GLSRVSD K HH+SDVL G++QGAL
hPAP-2a     IEYYICRGNAERVKE GRLSFYSGHSSFSMY CMLFVALYLQARMKG DWARLLRPTLQFGLV AVSIYVGLSRVSDYK HHWSDVLTGLIQGAL
hPAP-2b     IQNYRCRGDDSKVQE ARKSFFSGHASFSMY TMLYLVLYLQARFTW RGARLLRPLLQFTLI MMAFYTGLSRVSDHK HHPSDVLAGFAQGAL
hPAP-2c     QLEKVCRGNPADVTE ARLSFYSGHSSFGMY CMVFLALYVQARLCW KWARLLRPTVQFFLV AFALYVGYTRVSDYK HHWSDVLVGLLQGAL

              VI
            271         285 286         300 301         315 316
concensus   VA++ ++ +SD+FK  +               +
hPAP-2a     VAILVAVYVSDFFKE RTSFKERKEEDSHTT LHETPTTGNHYPSNH QP
hPAP-2b     VACCIVFFVSDLFKT KTTLSLPAPAIRKEI LSPVDIIDRNNHHNM M
hPAP-2c     VAALTVCYISDFFKA RPPQHCLKEEELERK PSLSLTLTLGEADHN HYGYPHSSS
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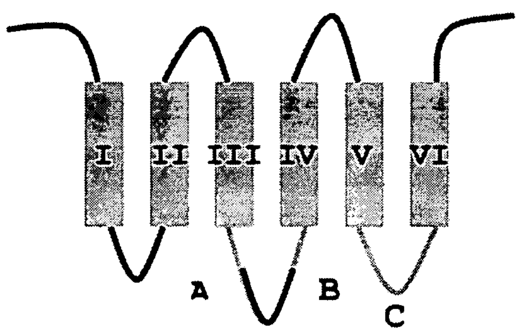

FIG. 1. **Sequence comparison of PAP-2a, -2b, and -2c.** The deduced amino acid sequences of PAP-2a, -2b, and -2c are shown with a consensus sequence in which conserved amino acid substitutions are indicated (+). *Boxed* regions denoted *A*, *B*, and *C* encompass regions of amino acid similarity that are conserved among other phosphatases as described under "Discussion." Regions of hydrophobic amino acids denoted *I–VI* are *underlined*. The *lower part* of the *figure* depicts the predicted transmembrane topology of the proteins.

concentrations of 3.2 mM Triton X-100 and 100 μM ^{32}P-labeled lipid substrate. For investigations of the dependence of enzyme activity on the surface concentration of substrate, the substrate concentration was 100 μM, and the detergent concentration was varied as indicated. Transfected cell fractions, Sf9 cell fractions, or detergent-extracted membrane proteins (generally 0.1–5 μg of protein) were added directly to these incubations. In some cases, the source of PAP activity was incubated with 5 mM NEM at 37 °C before addition to the incubations. Assays were performed at 37 °C and were terminated by the addition of ice-cold 10 mg/ml bovine serum albumin and 10% trichloroacetic acid. The samples were centrifuged for 5 min in a microcentrifuge, and [^{32}P]PO$_4^{2-}$ released into the supernatant was quantitated by liquid scintillation counting. This assay was validated by demonstrating that the water-soluble radioactivity released from the substrates was [^{32}P]PO$_4^{2-}$ by quantitative extraction with ammonium molybdate.

For assays of PAP activity using intact Sf9 cells, [^{32}P]dipalmitoyl-PA (approximately 10,000 dpm) was resuspended in unsupplemented Grace's medium containing 2 mg/ml bovine serum albumin to a final concentration of 20 mM by bath sonication. Assays were initiated by adding 1 ml of this substrate preparation to 1 ml of cells (generally 400 × 10^3 cells) and incubation at 37 °C with constant shaking. Aliquots of the suspension were removed at various times for determination of PAP activity by measurement of [^{32}P]PO$_4^{2-}$ release as described above. In some cases, samples were removed for determination of lactate dehydrogenase activity.

Other Methods—SDS-polyacrylamide gel electrophoresis, Western blotting, and protein determinations were performed as described previously (24). Lactate dehydrogenase activity was determined as described (25). Inorganic [^{32}P]PO$_4^{2-}$ was determined by extraction with ammonium molybdate (26).

RESULTS

Sequence Comparison of PAP-2a, -2b, and -2c—Fig. 1 shows an alignment of the deduced amino acid sequences of hPAP-2a, -2b, and -2c. PAP-2a comprises 289 amino acids corresponding to a protein of 32,788 kDa. PAP-2b comprises 311 amino acids corresponding to a protein of 35,120 kDa. PAP-2c comprises 288 amino acids corresponding to a protein of 32,577 kDa. PAP-2c is 54% identical to PAP-2a and 43% identical to PAP-2b. These values increase to 68 and 54%, respectively, when conservative amino acid substitutions are accounted for. It is notable that in comparison with PAP-2a and -2c, PAP-2b contains an extended N terminus with an enrichment of basic amino acid residues. The C-terminal 40 amino acids of the three proteins are also highly divergent. All three proteins contain a single consensus site for *N*-linked glycosylation (residue 140 in PAP-2c).

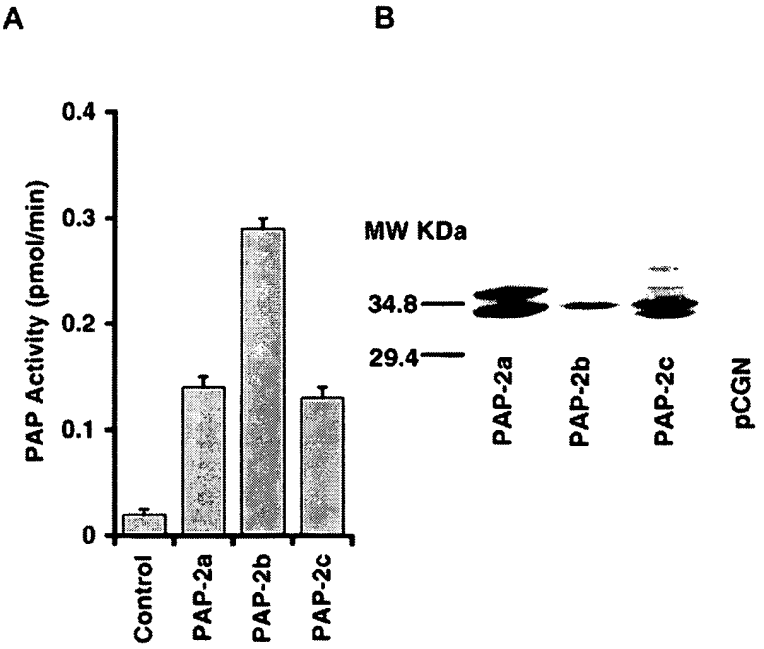

FIG. 2. **Expression of PAP-2a and PAP-2b.** *A*, PAP activity was determined in extracts prepared from HEK293 cells after transient transfection with pCGN PAP-2a, -2b, or -2c or pCGN. Data shown are means ± S.D. of triplicate determinations. *B*, proteins from cells transfected with pCGN PAP-2a, -2b, or -2c or pCGN were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel and analyzed by Western blotting as described under "Experimental Procedures." Molecular weights of prestained markers are shown. The data shown are representative of three separate experiments.

Hydropathy analysis of all three sequences suggests that PAP-2a, -2b, and -2c are integral membrane proteins with six membrane-spanning regions of 17–25 hydrophobic amino acid residues. These sequences are *underlined* in Fig. 1 and denoted as regions *I–VI*. Mutagenesis of rat PAP-2b supports this putative transmembrane topology (15).

Expression of PAP-2a, -2b, and -2c in HEK293 Cells—We expressed PAP-2a, -2b, and -2c as HA epitope-tagged proteins by transient transfection of HEK293 cells using a cytomegalovirus promoter-based vector. Cells were harvested 48 h post-transfection. PAP activity was determined in lysates of control and transfected cells using PA as substrate. The cells expressing PAP-2a, -2b, and -2c exhibited 7-, 15-, and 7-fold increases in membrane-associated PAP activity, respectively (Fig. 2*A*). Total protein from the transfected cells was separated by SDS-polyacrylamide gel electrophoresis on a 10% gel and analyzed by Western blotting using the HA epitope-specific 12CA-5 monoclonal antibody. In comparison with samples from untransfected cells, major immunoreactive proteins with estimated molecular masses of 33 and 37 kDa, 34 kDa, and 34 and 33 kDa were detected in cells expressing PAP-2a, -2b, and 2c, respectively (Fig. 2*B*). The immunoreactive material of higher molecular weight observed in the case of PAP-2a and -2c presumably represents the glycosylated forms of the proteins. We found that immunoreactive species of lower mobility could be partially converted to the faster migrating species upon treatment of membrane extracts with *N*-glycanase prior to SDS-polyacrylamide gel electrophoresis. PAP-2b also contains a consensus site for *N*-linked glycosylation, and we occasionally observed a minor immunoreactive species of higher molecular weight upon expression of this cDNA in COS-7 and HEK293 cells (not shown). It is unclear why comparatively higher levels of activity were observed in the pCGN PAP-2b transfected cells. It is possible that PAP-2b has an intrinsic higher specific activity than the other enzymes under the assay conditions we used or that this enzyme is differentially regulated in a manner that produces a specific increase in activity relative to the other enzymes.

Expression of PAP-2a, -2b, and -2c in Insect Cells Using Baculovirus Vectors—Recombinant baculovirus-infected Sf9 insect cells have proved to be an effective system for studying the regulation of integral membrane signaling proteins including adenylyl cylase isoforms and G-protein-coupled receptors (27, 28). In comparison with the transient transfection studies described above, this approach also provides a simple and consistent means to produce recombinant protein that is necessary for detailed investigations of the catalytic properties of these enzymes. Recombinant baculoviruses for expression of PAP-2a, -2b, and -2c were prepared and monolayer cultures of insect cells infected with these viruses and a virus expressing a control protein (murine PLD2) as described under "Experimental Procedures." Cells were harvested 48 h post-transfection and fractionated into cytosolic and membrane fractions. PAP activity in these fractions was determined using PA as substrate. In comparison with control cells, cells expressing PAP-2a, -2b, and -2c exhibited dramatic (1140-, 540-, and 460-fold) increases in membrane-associated PAP activity (Table I). Cytosolic PAP activity was unaltered in cells expressing PAPs-2a, -2b, and -2c. PAP activity could be effectively extracted from the Sf9 cell membranes by a combination of 1% Triton X-100 and 1% β-D-octylglucopyranoside. This procedure routinely solubilized 70–80% of PAP-2a, -2b, and -2c activity and approximately 30% of total membrane protein. Type 2 PAP activities are characteristically independent of Mg^{2+} and insensitive to inhibition by NEM. Our standard assay medium contains 2 mM EDTA and no added Mg^{2+}. Activity of the extracted PAP-2a, -2b, and -2c was unchanged by the addition of $MgCl_2$ to the assays to give a Mg^{2+} concentration of 5 mM. Similarly, preincubation of the extracted material with 5 mM NEM produced very modest decreases in PAP-2a, -2b, and -2c activity (Table I). Baculovirus-infected Sf9 cells are therefore an excellent model system for investigating the catalytic properties of recombinantly expressed PAP-2 enzymes.

Cell Surface Activity of PAP-2a, -2b, and -2c in Baculovirus-infected Insect Cells—Cell surface PAP activity has been detected in many different cell types, but the identity of the enzyme responsible has not been determined. To investigate the cell-surface expression of PAPs-2a, -2b, and -2c, we measured PAP activity of intact baculovirus-infected insect cells expressed using [^{32}P]dipalmitoyl-PA presented in 1 mg/ml bovine serum albumin. Fig. 3 shows time courses of PAP activity determined as ^{32}P release measured using intact cells or equiv-

TABLE I

Expression of PAP-2a, -2b, and -2c in insect cells using baculovirus vectors

Monolayer cultures of Sf9 cells were infected with recombinant baculoviruses for expression of PAP-2a, -2b, and -2c and a control protein (murine PLD2). The cells were harvested, lysed, and fractionated as described under "Experimental Procedures." PAP activity was determined using PA as substrate under the conditions indicated (see "Experimental Procedures" for details). The data shown are means ± S.E. of data from three independent experiments.

	PAP activity			
	Control	PAP-2a	PAP-2b	PAP-2c
	pmol/min/mg protein			
Membrane	0.05 ± 0.001	57 ± 3	27 ± 2	23 ± 2
Cytosol	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.04	0.2 ± 0.04
Detergent Extract	0.25 ± 0.04	228 ± 22	167 ± 21	123 ± 12
Detergent Extract + Mg^{2+}	0.3 ± 0.05	220 ± 23	158 ± 30	128 ± 11
Detergent Extract + NEM	0.2 ± 0.04	191 ± 9	147 ± 11	115 ± 14

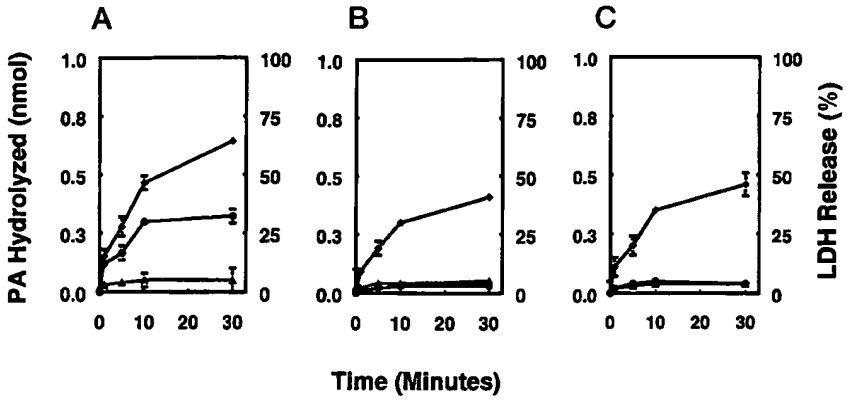

FIG. 3. **PAP activity in intact insect cells.** Monolayer cultures of Sf9 cells were infected with recombinant baculoviruses for expression of PAP-2a (*A*), -2b (*B*) and -2c (*C*). The cells were harvested, washed, and resuspended in unsupplemented Grace's medium as described under "Experimental Procedures." PAP activity was determined using PA as substrate in samples of intact cells (●) or cells that had been disrupted by sonication (♦). Lactate dehydrogenase release from the cells was determined as described under "Experimental Procedures" and expressed as a percentage of activity in cells that had been disrupted by sonication (▲). The data shown are means ± S.D. of triplicate determinations and are representative of three separate experiments.

alent quantities of cells that have first been disrupted by sonication. Cell integrity was determined by measurement of lactate dehydrogenase release in parallel incubations. As expected, sonicated dispersions of PAP-2a-, PAP-2b-, and PAP-2c-expressing Sf9 cells exhibited high levels of PAP activity. Intact Sf9 cells expressing PAP-2a but not PAP-2b or -2c exhibited significantly elevated levels of PAP activity. In comparison with the sonicated cells, 60% of total Sf9 cell PAP activity could be measured in intact cells expressing PAP-2a, while only 8 and 10% of total PAP activity was detected in intact cells expressing PAPs -2b and -2c. Lactate dehydrogenase release after these 30-min incubations varied between 2 and 5%, indicating that the majority of the cells were intact and that the observations of PAP activity could not be accounted for by cell lysis during the incubation period. One possible explanation for these results is that the PA substrate is accumulated by the cells and metabolized at an intracellular site. This did not appear to be the case, since no appreciable radioactivity became associated with the cells upon incubation with [^{32}P]PA, and the kinetics of substrate hydrolysis measured as decrease in [^{32}P]PA paralleled the release of [^{32}P]PO_4^{2-} (data not shown).

Kinetic Analysis of PAP-2a, -2b, and -2c Activity against Substrates Presented in Nonionic Detergent Micelles—Purified preparations of rat liver type 2 PAP activity have been reported to hydrolyze C1P and S1P in addition to PA, while transient expression of PAPs-2a and -2b resulted in an increased hydrolysis of both PA and C1P with the 2b isoenzyme exhibiting an apparent selectivity for S1P. Measurements of the affinities and maximal rates of substrate hydrolysis by PAPs-2a, -2b, and -2c would provide useful insight into their likely biological functions. Kinetic analyses of lipolytic or lipid-modifying enzymes are best made using substrates presented in micelles of detergents with high aggregation numbers, where the surface concentration of substrate can be varied without compromising the physical state of the micelles. Other workers have usefully employed a surface dilution kinetic model (29) in which substrates are presented as Triton X-100 micelles for analysis of yeast and liver type 2 PAP activities (11, 30). Clearly, these types of studies would best be performed using purified enzymes, but we were encouraged that the extremely high levels of expression obtained with the baculovirus system suggested that the use of detergent-extracted recombinant PAP enzymes in these assays would minimize interference from contaminating activities. We first examined the activity of PAP-2a, -2b, and -2c against PA, C1P, and S1P presented as components of Triton X-100 or dodecyl β-D-maltoside micelles. The substrate concentration was 100 mM, and the detergent concentration was 3 mM. The results obtained are shown in Table II. All three PAP enzymes were highly active against PA presented in both detergents. PAP-2a, -2b, and -2c also hydrolyzed C1P with high activity. For PAP-2a and -2b, activity with C1P as substrate was 53 and 72% of that observed with PA, whereas for PAP-2c, activity was 130% higher with C1P as substrate. The comparable activities of the enzymes against substrates presented in micelles of two structurally different detergents suggested that the apparent differences in substrate utilization reflected true enzyme specificity rather than a selective effect of the detergent on substrate presentation to the enzyme.

To calculate kinetic constants, we determined the activity of PAP-2a, -2b, and -2c using mixed micelles of Triton X-100 and PA, LPA, C1P, or S1P substrates. Initial rates of hydrolysis were measured as the surface concentration of each substrate

TABLE II
Substrate specificity of PAP-2a, -2b, and -2c

Detergent-extracted membrane proteins from Sf9 cells expressing PAPs-2a, -2b, or -2c or a control protein (mPLD2) were incubated with the indicated substrates at concentrations of 100 μM in the presence of 3 mM Triton X-100 or 3 mM dodecyl β-D-maltoside, and hydrolysis was measured as initial rate of $[^{32}P]PO_4^{2-}$ release. The data shown are means ± S.E. of data from three independent experiments.

	Substrate hydrolysis							
	Control		PAP-2a		PAP-2b		PAP-2c	
	PA	C1P	PA	C1P	PA	C1P	PA	C1P
	pmol/min/mg protein							
Triton X-100	0.5 ± 0.01	0.1 ± 0.02	320 ± 12	170 ± 14	220 ± 28	160 ± 24	90 ± 8.1	120 ± 17
Dodecyl-β-D-maltoside	0.6 ± 0.02	0.1 ± 0.01	413 ± 32	189 ± 21	244 ± 22	174 ± 21	128 ± 12	147 ± 24

was increased, and the data obtained are shown in Fig. 4. For all three enzymes, activity increased with increasing substrate concentration in an apparently saturable manner, suggesting that a surface dilution kinetic model was appropriate for analysis of these enzymes. The data were analyzed by weighted nonlinear regression fit to the equation $V = V_{max} A/(K_m + A)$ where V is the initial rate and A is the substrate concentration in mol % to calculate V_{max} and K_m values. R values for this analysis ranged from 0.84 to 0.97. The results obtained are presented in Table III.

Inhibition of PAP-2a, -2b, and -2c Activity by Sphingosine, Zn^{2+}, and Propranolol—The product of S1P hydrolysis by PAP, sphingosine, has been reported to be a potent inhibitor of several type 2 PAP activities. PAP-2a, -2b, and -2c activity was determined using PA as substrate in the presence of increasing concentrations of sphingosine. All three enzymes were inhibited by this compound. Half-maximal inhibition of the 2a and 2b isoenzymes was observed at approximately 350 mM sphingosine, while the 2c isoform was appreciably more sensitive to this compound with half-maximal inhibition observed at approximately 100 mM (Fig. 5*A*). Several other inhibitors of type 2 PAP activities have been described and used to modulate PAP activity both *in vitro* and in intact cells. Zn^{2+} was an effective inhibitor of all three enzymes with half-maximal inhibition observed at 30 mM (Fig. 5*B*). By contrast, propranolol, which is an effective inhibitor of type 1 PAP activities was only modestly effective as an inhibitor of PAP-2a, -2b, and -2c, with the -2b isoform appearing somewhat more sensitive to inhibition by this agent (Fig. 5*C*).

DISCUSSION

PAP-2c is a third member of the family of PAP-2 isoenzymes. This enzyme shares the putative transmembrane topology of the other PAP-2 isoenzymes, having six regions of predominantly hydrophobic amino acids linked by extramembrane regions. This proposed transmembrane structure is similar to that of the membrane-spanning portions of membrane-bound adenylylcyclases and transport proteins of the P-glycoprotein superfamily, which consist of a short N terminus and two transmembrane regions consisting of six hydrophobic spans that link globular cytoplasmic domains (28). Comparison of the sequences of hPAP-2a and hPAP-2b with previously recognized homologs identifies three regions of conserved sequence (denoted as *A*, *B*, and *C* in Fig. 1), which contain invariant amino acids that define a signature sequence motif shared among several proven or putative lipid phosphatases, the mammalian glucose 6-phosphatases, and some bacterial nonspecific and acid phosphatases. This group also contains yeast and *Drosophila* PAP-2 homologs (13–20). These conserved regions lie predominantly within the hydrophilic regions of the proteins, and homologous sequences are also found in a soluble globular proteins that include bacterial acid phosphatase, mammalian glucose 6-phosphatase, and a fungal vanadium-dependent chloroperoxidase (29). The structure of this latter enzyme has been recently determined (30). The pentacoordinate vanadate cofactor resembles the transition state structure of phosphate. This suggests a two-step reaction in which a charge relay system involving conserved His and Asp residues in region C establishes a histidine-phosphate bond and the catalytic histidine residue in region B acts as an acid to cleave this intermediate, releasing the dephosphorylated substrate. This residue then acts as a base to facilitate nucleophilic attack on the phosphohistidine intermediate by a water molecule. Catalytically inactive PAP-2 mutants would be valuable tools for *in vivo* studies. We found that substitution of Ser^{174} with alanine in PAP-2a produces a catalytically inactive protein.[2] This residue is postulated to form a hydrogen bond to the phosphate atom of the substrate, stabilizing the pentacoordinate transition state structure during catalysis.

Like PAP-2a and PAP-2b, PAP-2c is exclusively membrane-bound when expressed in mammalian or insect cells. As with many integral membrane proteins, purification of the type 2 PAP enzymes has been difficult. We made baculoviruses for expression of PAP-2a, -2b, and -2c and found that baculovirus-infected insect cells were an excellent model system for generating recombinant PAP enzymes. These cells exhibited extremely low levels of endogenous PAP activity. Infection with appropriate viruses resulted in dramatic increases in PAP activity. Membranes or detergent-extracted membrane proteins from these cells provided an abundant source of activity for kinetic studies. The Sf9 cell system should be effective for investigating the regulation of the type 2 PAP enzymes and may also provide an appropriate source for purification of recombinant proteins.

We used Triton X-100 mixed micelles to investigate the substrate specificity of PAP-2a, -2b, and -2c. The activity of all three enzymes was dependent on the surface concentration of substrates, suggesting that a surface dilution kinetic model was appropriate for analyzing the kinetic behavior of the enzymes in this model system. According to this model, which was originally developed for studies of phospholipase A_2, enzyme activity depends on both bulk and surface concentrations of substrate, because enzyme binding to the micelle interface precedes substrate binding and catalysis (31, 32). Since the PAP-2 enzymes are clearly integral membrane proteins, their association with detergent micelles would be expected to be effectively irreversible, so this type of analysis can be simplified to measurements of the dependence of enzyme activity on the surface concentration of substrate. The major limitation of this approach is that substrate or enzyme molecules can exchange between detergent micelles, and for highly active enzymes this rate of substrate exchange can be limiting (33). It is also possible that the detergent employed for these measurements may have selective affects on enzyme activity, although our finding that the enzymes displayed similar profiles of activity with

[2] R. Roberts and A. J. Morris, unpublished results.

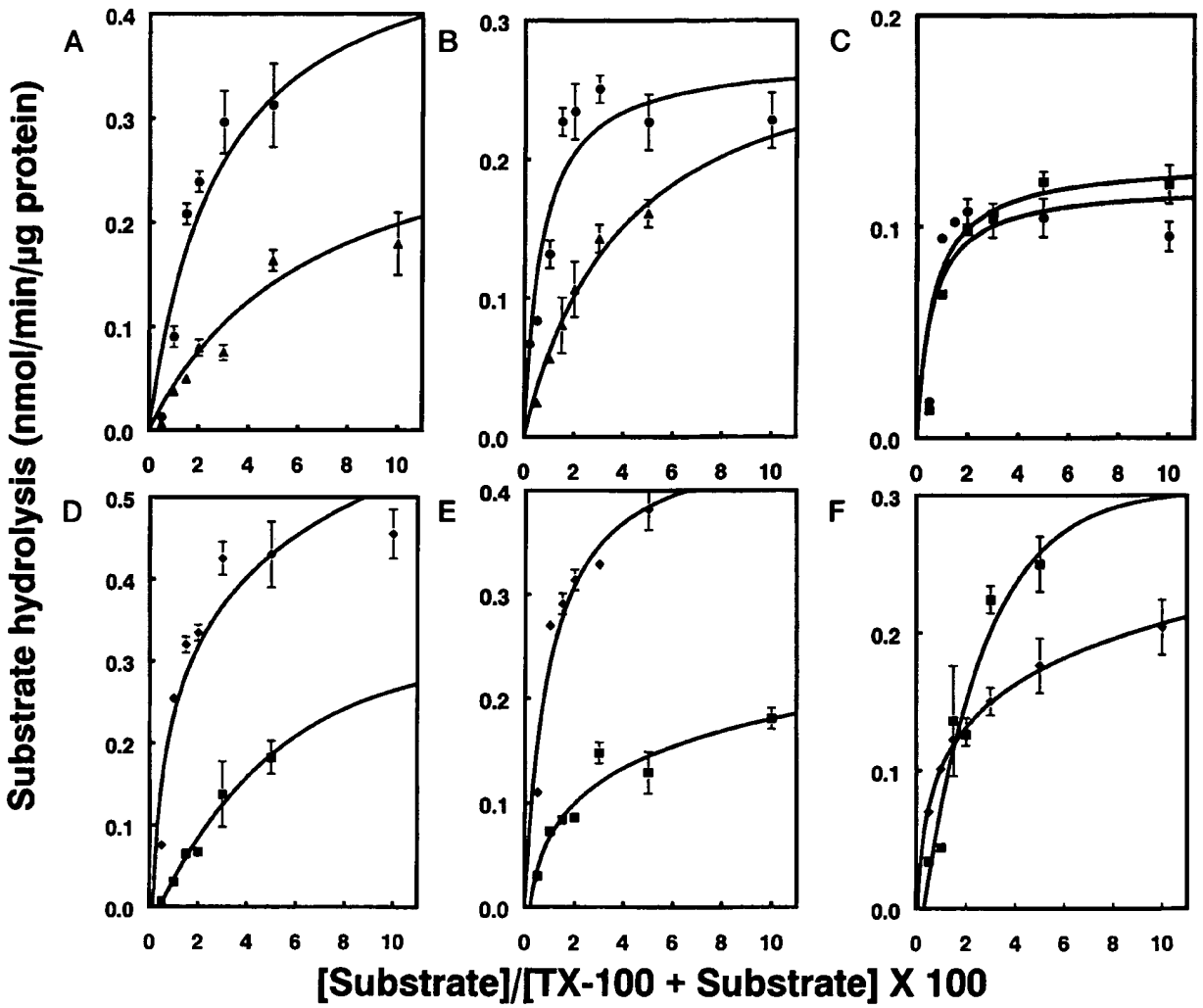

FIG. 4. **Surface concentration dependence of PAP activity in TX-100 micelles.** Activity of Sf9 cell-expressed detergent-extracted PAP-2a (*A* and *D*), -2b (*B* and *E*), and -2c (*C* and *F*) was determined by measuring initial rates of release of $[^{32}P]PO_4^{2-}$ as the mol fraction of PA (●), C1P (▲), S1P (■), or LPA (◆) was increased as indicated. The data shown are means ± S.D. of triplicate determinations and are representative of three independent experiments.

TABLE III

Specificity of PAP-2a, -2b, and -2c for substrates presented in Triton X-100 micelles

V_{max} (nmol/min/mg) and K_m (mol %) values were determined as described under "Results." The data shown are means ± S.E. of data obtained from three independent experiments.

	PAP-2a			PAP-2b			PAP-2c		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
PA	0.54 ± 0.03	3.4 ± 0.05	0.16	0.27 ± 0.05	0.61 ± 0.03	0.44	0.15 ± 0.02	0.44 ± 0.07	0.34
LPA	0.50 ± 0.05	1.3 ± 0.03	0.39	0.46 ± 0.03	1.11 ± 0.30	0.41	0.20 ± 0.04	1.35 ± 0.23	0.15
C1P	0.30 ± 0.04	7.1 ± 1.1	0.04	0.36 ± 0.1	3.4 ± 0.02	0.11	0.17 ± 0.03	0.78 ± .021	0.22
S1P	0.32 ± 0.10	2.8 ± 0.4	0.11	0.24 ± 0.09	2.5 ± 0.02	0.10	0.69 ± 0.10	5.99 ± 1.92	0.12

substrates presented in Triton X-100 or dodecyl β-D-maltoside micelles argues against this possibility. The data from these experiments were used to calculate V_{max} and K_m values for the different substrates. Although the extremely high levels of expression obtained with the insect cell system rendered interference from endogenous activities insignificant, because the enzyme preparations used were not pure, comparison of the absolute V_{max} values of the enzymes is not meaningful. The data obtained do, however, provide a quantitative comparison of the relative activities of the individual PAP enzymes for the different substrates tested. Our results indicate that PAP-2a, -2b, and -2c can hydrolyze PA, LPA, C1P, and S1P and that there are differences in the selectivity of the enzymes for these four substrates. PAP-2a displayed comparable V_{max} values for all four substrates, with highest activity displayed against LPA and PA. PAP-2b showed a similarly higher relative V_{max} activity with LPA as substrate, while PAP-2c displayed significantly higher activity against sphingosine phosphate. K_m values for the substrates varied more widely. Specificity constants (V_{max}/K_m) provide a means to quantitate the specificity of the three enzymes for the different substrates. These values are given in Table III, and they indicate a relative rank order of substrate selectivity of LPA > PA > S1P > C1P for PAP-2a, LPA ≈ PA > C1P > S1P for PAP-2b, and PA > C1P > LPA > S1P for

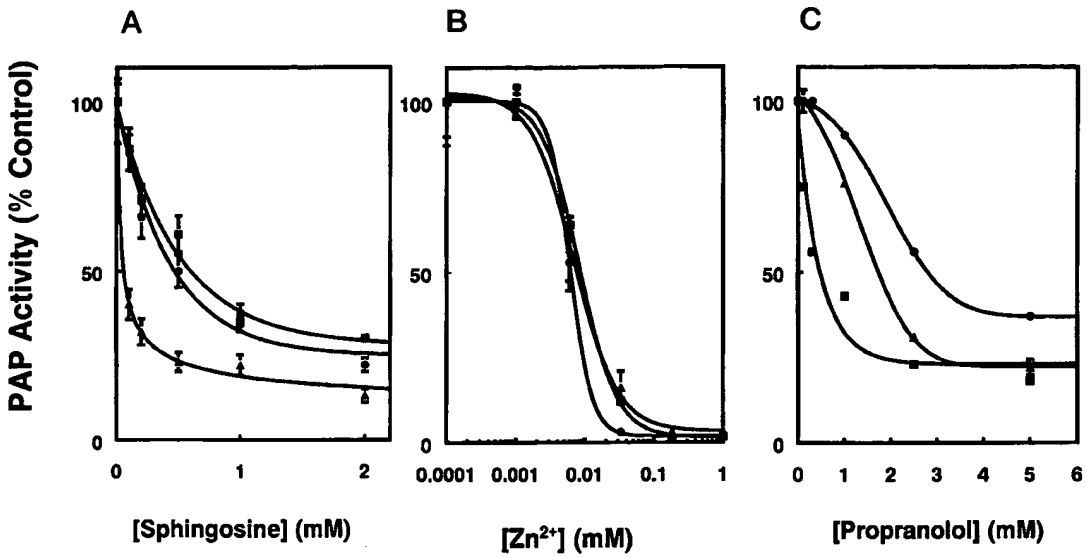

FIG. 5. **Inhibition of PAP-2a, -2b, and -2c by sphingosine, Zn^{2+}, and propranolol.** Activity of Sf9 cell-expressed detergent-extracted PAP-2a (●), -2b (▲), and -2c (■) was determined using PA as substrate in the presence of the indicated concentrations of sphingosine (*A*), $ZnCl_2$ (*B*), or propranolol (*C*). The data shown are means of triplicate determinations ± S.D. expressed a percentage of activity determined in the absence of any inhibitor. The data shown are representative of three separate experiments.

PAP-2c. The K_m values obtained with PA as substrate are comparable with those reported for a yeast PAP (32) but somewhat higher than previously determined for a liver form of the enzyme (11). Our results are in broad agreement with studies that used transiently transfected HEK293 cells as a source of enzyme activity to demonstrate that PAP-2a and -2b could hydrolyze PA, C1P, and S1P, although we did not observe the selectivity of PAP-2b for S1P reported by these investigators (13, 14).

The diversity of PAP-2 enzymes revealed by cDNA cloning was somewhat unexpected, since purification studies suggested that this enzyme behaved as a single activity from a number of different mammalian tissues (4–8). PAP-2 activity has been studied extensively in rat liver, where a highly purified enzyme preparation has been reported to hydrolyze PA, C1P, and S1P with comparable avidity. The finding that each substrate inhibited the hydrolysis of the other in a competitive manner further supports the contention that this PAP-2 enzyme hydrolyzes all three substrates (11). The relationship of the rat liver PAP-2 enzyme to the cloned isoenzymes is not known, and this issue clearly requires further investigation. The *S. cerevisiae* genome contains four open reading frames encoding proteins with extensive homologies to the type 2 PAPs. Recent work has identified a member of one class as a PA and diacylglycerol pyrophosphate phosphatase and the members of the remaining class as S1P phosphatases (18–20). PA and C1P did not inhibit the hydrolysis of S1P by these yeast enzymes (19), suggesting that they are highly specific for S1P. In comparison with mammalian PAP-2a, -2b, and -2c, the yeast S1P phosphatases have several unique structural features including an extended C terminus and several nonconservative substitutions of amino acid residues around the presumed active site. We have identified a cDNA encoding a fourth human type 2 PAP enzyme, which has a number of provocative homologies to the yeast S1Pases.[2] This may prove to be a mammalian S1P-selective PAP-2 isoenzyme.

Fractionation studies using rat hepatocytes indicate that PAP-2 activity is enriched in the plasma membrane, and recombinantly expressed mouse PAP-2a is also localized to this compartment in HEK 293 cells (4, 13). Several cells have been reported to express a cell surface PAP activity (9, 10). We measured PAP activity in intact Sf9 cells expressing PAPs-2a, -2b, and -2c. The results obtained clearly indicate that when expressed in these cells, the active site of PAP-2a has effective access to extracellular substrates. The simplest explanation for these results is that this is an ectoenzyme that is oriented in the plasma membrane with its active site facing the extracellular space. Although we cannot rule out the possibility that a highly active "flippase" delivers PA substrate to this enzyme at an intracellular site, we consider this possibility unlikely because hydrolysis of PA by PAP-2a-expressing insect cells was rapid and proceeded without significant accumulation of radiolabeled substrate by the cells. PA, LPA, and S1P are receptor-active compounds, and it is probable that one function of PAP-2a is to terminate the signaling functions of these lipid agonists. Studies using fluorescent PA analogs reveal that dephosphorylation of PA to form DG precedes DG uptake into the cell. Cell surface PAP-2a may also provide a mechanism for formation of DG from extracellular substrates (34). Unfortunately, immunocytochemical analysis reveals that the HA-tagged PAP-2a fails to exit the endoplasmic reticulum when expressed in HEK293 cells, so we have therefore not yet been able to examine cell surface PAP activity in mammalian cells expressing this enzyme.[3]

As discussed above, the PAP-2 enzymes appear to be glycoproteins that share the same predicted transmembrane topology. Our results suggest that, in comparison with PAP-2a, PAP-2b and -2c are localized to different intracellular membrane compartments. Clearly, localization studies, preferably using PAP-2 isoenzyme-selective antibodies to study endogenous enzymes, are needed to further define the subcellular distribution of the PAP-2 isoenzymes. Rat PAP-2b is localized to the endoplasmic reticulum in cultured rat intestinal epithelial cells (15). A portion of type 1 PAP activity in rat liver co-localized with endoplasmic reticulum markers during cell fractionation studies (4, 35). Despite this apparent co-localization, PAP-2b is clearly a type 2 PAP enzyme, and presumably the endoplasmic reticulum-localized PAP-1 activity reported in

[3] V. A. Sciorra and A. J. Morris, unpublished observations.

rat liver represents the product of a separate gene. These findings are particularly interesting in light of the growing body of information that points to a role for PLD-generated PA in controlling vesicular transport between the endoplasmic reticulum and Golgi apparatus as well as the cis and trans compartments of the Golgi. PA has been proposed to play an essential role in recruitment of cytosolic coatomer complexes to the surfaces of the respective membrane compartments involved in these transport processes, which in turn initiates formation of coated transport vesicles (36–38). Such a mechanism would require tight control of PA levels, and localization of PAP activity to this membrane compartment may therefore play an important role in this process.

Clearly, establishment of the roles played by the PAP-2 enzymes in cellular lipid metabolism remains an important priority. Our results suggest roles for the PAP-2 enzymes in the metabolism of both phospholipid- and sphingolipid-derived signaling molecules. Further work is required to establish the physiological role of these enzymes in metabolism of these bioactive lipid substrates. In this regard, it is noteworthy that PAP activities appear to act in series with PLD to generate and interconvert PA and diacylglycerol. PLD1 and PLD2, two recently identified mammalian PLD enzymes, are localized to different subcellular compartments in fibroblasts. PLD1 is found in a perinuclear compartment (most likely representing the endoplasmic reticulum or Golgi), while PLD2 is localized to the plasma membrane (39). These findings raise the possibility that the PAP-2 enzymes play selective roles in signaling processes controlled by these two PLDs, and future work will be directed toward investigating this possibility.

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Note Added in Proof—While this paper was in press, Hooks and co-workers (Hooks, S. B., Ragan, S. P., and Lynch, K. R. (1998) *FEBS Lett.* **427,** 188–192) also reported the cloning and expression of a human PAP-2c cDNA.

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